Familial melanoma-associated mutations in p16 uncouple its tumor suppressor functions

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Familial melanoma is commonly associated with point mutations in the cyclin-dependent kinase (CDK) inhibitor p16INK4A (p16). p16 is thought to function as a tumor suppressor by negatively regulating the cell cycle through binding CDK4; however, several melanoma-associated p16 mutations do not compromise CDK4-binding. We recently identified a novel role for p16 in regulating intracellular oxidative stress that is independent of cell cycle control. Here, we constructed 13 different familial melanoma-associated point mutants spanning the p16 coding region and analyzed their capacity to regulate cell-cycle phase and reactive oxygen species (ROS). Compared to wild-type p16 which fully restored both functions in p16-deficent cells, various p16 mutants differed in their capacity to restore ROS and cell cycle profiles. While some mutations (R24P, G35A, G35V) did not impair either function, others (P81T, R87W, L97R, A148T) impaired both. Interestingly, several predominantly impaired cell-cycle (R24Q, R99P, V126D) or oxidative function (A36P, A57V, P114S), indicating that these two functions of p16 can be uncoupled. Examination of the p16 crystal structure revealed that some mutations impairing both cell-cycle and oxidative functions (P81T, R87W, L97R), or only cell-cycle function (R99P), localize to the third ankyrin repeat containing the putative CDK4-binding site. On the other hand, most mutations impairing cell-cycle but not oxidative function (A36P, A57V, P114S), or those not impairing either function (R24P, G35A, G35V), lie outside this region. These results demonstrate that familial melanoma-associated point mutations in p16 can selectively compromise these two independent tumor-suppressor functions (cell cycle and oxidative regulation), which may be mediated by distinct regions of the protein.

A mixture of Schinus terebinthifolius Raddi extract and linoleic acid from Passiflora alata oil synergically decreases the level of melanin synthesis in human reconstituted epidermis

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Two botanical compounds coming from Brazilian biodiversity, an extract of Schinus terebinthifolius Raddi and a linoleic acid fraction isolated from Passiflora alata oil, were developed as alternative and safer technology for skin whitening, once classical drugs like hydroquinone, arbutin and kojic acid have been described to show a carcinogenic potential. The whitening effect of these compounds was assessed using biochemical assays and *in vitro* models including cellular culture and equivalent skin. The results showed that these natural compounds are able to reduce until 89.9% of the tyrosinase activity *in vitro* as well as to decrease 20% the level of melanin produced by B16 cells previously treated with melanocyte-stimulating hormone ($p<0.05$). Furthermore, melanin was 23.0 % reduced in tanned human reconstituted epidermis models treated with the compounds ($p<0.01$). The combination of the compounds provided a synergic positive whitening effect rather than their isolated use. Finally, we demonstrated that the performance of these mixed compounds is comparable to classical molecules used for skin whitening, as kojic acid. This new natural mixture is not carcinogenic or cytotoxic and would be useful as a therapeutic agent for treating hyperpigmentation and as an effective component in whitening cosmetics.

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Plexin C1, a neural guidance receptor, suppresses melanoma progression

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Our purpose was to examine the role of Plexin C1 (PC1) receptor on melanoma progression. PC1 regulates neural guidance and immune function, has Ras-GAP activity, is expressed by human melanocytes, and regulates melanocyte cytoskeletal architecture and cellular adhesion. Our previous data showed that PC1 is lost in melanoma *in vivo*. We hypothesized that introduction of PC1 would suppress melanoma progression. We introduced PC1 into melanoma, and silenced Plexin C1 in melanocytes, and examined proliferation, migration, apoptosis, and tumor growth in mice. PC1 expression profoundly inhibited melanoma proliferation; conversely, silencing of PC1 in melanocytes stimulated melanocyte proliferation 2-fold.To determine targets that contribute to suppression of melanoma proliferation, we examined PCNA and cyclin D1, and P-Erk1/Erk2. None were regulated by PC1 in melanoma and PC1 did not drive melanoma into senescence, as evaluated by β-galactosidase staining. Melanoma cells expressing PC1 migrated significantly slower compared with LacZ controls, whereas PC1 silenced melanocytes showed significantly increased migration. Analysis of effects of PC1 on resistance to cisplatin induced apoptosis in melanoma revealed a protective effect of PC1, which was due to sustained activation of the PI3-kinase-Akt pathway. Finally, melanoma from two cell lines (C32 and WM115) expressing PC1 were injected into the flanks of NOD.CgPrkdscid mice. PC1 significantly delayed tumor initiation (C32) and tumor size (WM115) at 3 weeks and 8 weeks respectively. These data are important because they demonstrate a potential role for PC1 as a tumor suppressor protein for melanoma. We speculate that increased Ras-GAP activity secondary to PC1 signaling suppresses melanoma progression through effects on cytoskeletal regulation. This may be counterbalanced by PC1-dependent Akt activation, resulting in enhanced tumor survival.

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Characterization of the effects of BRAF inhibitors on melanoma metabolism *in vivo* and *in vitro*

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Vemurafenib is the first FDA-approved personalized treatment for patients with BRAF V600E-positive metastatic melanoma to show a survival benefit. Most patients show significant tumor shrinkage within weeks of the initiation of therapy. Resistance to the drug, however, eventually develops in many patients. Understanding the biological effects of vemurafenib and the underlying mechanisms of resistance is crucial to develop therapeutic strategies that can further improve survival and quality of life in patients with metastatic melanoma. Biomarker and phenotypic correlates of response are difficult to determine in humans because generally only one on-treatment biopsy specimen is available for comparison to a pre-treatment specimen. In contrast, an ideal animal model system would allow for a thorough evaluation of the time course and phenotype of BRAF inhibitor-response and subsequent relapse. The conditional BRAF/PTEN mouse model of melanoma that we co-developed is such a system. In this model, melanocyte-specific expression of BRAF V600E and loss of PTEN is under control of the Cre-Lox recombination system. Activation of the Cre recombinase-estrogen receptor fusion protein requires topical application of 4-hydroxytamoxifen, allowing for induction of recombination at specific time points in the life span of the mouse. Metabolic alterations, such as the switch from oxidative phosphorylation to aerobic glycolysis that is associated with malignant transformation (Warburg effect), are important for cancer development. Similarly, metabolic changes in cancer cells in response to treatment may be important for the mechanism of action and effectiveness of oncologic drugs. For these reasons, specific studies include evaluation of the effect of PLX4720, a vemurafenib analog, on the expression, localization, and activation of proteins involved in glucose metabolism in both the BRAF/PTEN mouse model and cell culture.

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Melanosome biogenesis protein complexes interact with the endosomal WASH complex

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Melanin pigment is synthesized in specialized lysosome-related organelles termed melanosomes. Biogenesis of melanosomes requires the targeting of specific membrane proteins and soluble cargoes to maturing melanosomes. One mechanism for this targeting is the recruitment and concentration of cargo into nascent vesicles by adaptor protein complexes, such as the adaptor protein complex-3 (AP-3). Mutations to AP-3 and related protein complexes including the BLOC-1 complex cause the Hermansky Pudlak Syndrome, which is characterized by hypopigmentation as well as platelet dysfunction and pulmonary fibrosis. Adaptor proteins are recruited to membranes from the cytosol in part by specialized phospholipids, making lipid kinases key regulators of membrane traffic. One such regulator is the phosphoinositol-4-kinase type IIα (PI4KIIα), which is known to bind and regulate the function of both AP-3 and BLOC-1 complexes. In order to further our understanding of the mechanism by which PI4KIIα regulates cargo delivery by AP-3 and BLOC-1 complexes, we performed immunoaffinity chromatography of PI4KIIα protein complexes coupled to *in vivo* isotope tagging of the cell proteome and mass spectrometry (SILAC). We identified several regulators of the actin cytoskeleton, including the WASH complex, an Arp 2/3 activator, and the RhoA guanine exchange factor RhoGEF1. We confirmed these interactions by independent immunoaffinity chromatography experiments, sucrose velocity sedimentation, and deconvolution immunofluorescent microscopy. We conclude that PI4KIIα, a key enzyme regulating cytosolic coats required for melanosome biogenesis, interacts with RhoGEF1 and the WASH complex. Future work will test the hypotheses that the WASH complex is required for AP-3- and/or BLOC-1-dependent vesicle sorting and fission for melanosome biogenesis.

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The association of PTPN22 promoter variation and altered T cell cytokine production with vitiligo in Chinese Han population

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Protein tyrosine phosphatase, non-receptor type 22 (PTPN22) is a protein tyrosine phosphatase and a negative regulator of the T cell response. Recent studies have suggested other polymorphisms besides 1858 C>T polymorphism in the PTPN22 gene may be associated with autoimmune diseases in Asian populations. In this hospital-based case-control study of 749 vitiligo patients and 763 matched controls, we investigated 2 PTPN22 polymorphisms (rs2488457 G>C and rs3811021 A>G) to determine whether these are associated with vitiligo susceptibility in the Chinese population. In addition, the T cells profile and function were investigated to evaluate possible associations between the PTPN22 promoter polymorphism rs2488457C allele and clinical and laboratory findings of vitiligo.We found that an increased risk of vitiligo was associated with the PTPN22rs2488457 C variant allele.No evidence for any association between the rs3811021 polymorphism and vitiligo susceptibility was found. In addition, a significantly increased IFN-γ cytokine production of T cell was observed in the rs2488457 C allele vitiligo patients. Our findings suggested that the PTPN22 promoter polymorphism rs2488457 G>C is associated with T cell IFN-γ production and that there exists a genetic predisposition for vitiligo in the Chinese population.

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Epidermal growth factor facilitates melanoma lymph node metastasis by influencing tumor lymphangiogenesis

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Alterations in epidermal growth factor (EGF) expression have been described to be of prognostic relevance in human melanoma but EGF mediated effects on melanoma have not been extensively studied. Since lymph node metastasis usually represents the first major step in melanoma progression, we were trying to identify a potential role of primary tumor-derived EGF in the mediation of melanoma lymph node metastases. Stable EGF-knockdown (EGFkd) in EGF-high (M24met) and EGF-low (A375) expressing melanoma cells was generated. Only in EGF-high melanoma cells, EGFkd had observable effects by significantly reducing lymph node metastasis and primary tumor lymphangiogenesis *in vivo* as well as impairing tumor cell migration *in vitro*. Moreover, EGF induced sprouting of lymphatic but not of blood endothelial cells was abolished using supernatants of M24met EGFkd cells. Additionally, M24met EGFkd tumors showed reduced VEGF-C expression levels. Similarly, in human primary melanomas, a direct correlation between EGF/VEGF-C and EGF/Prox-1 expression levels was found. Finally, melanoma patients with lymph node micrometastases undergoing sentinel-node biopsy were found to have significantly elevated EGF serum levels as compared with SLN negative patients. Our data indicate that tumor-derived EGF is important in mediating melanoma lymph node metastasis.

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A counter-intuitive role for apoptotic caspase 3 in treatment of melanoma and other cancers

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In cancer treatment, apoptosis is a well-recognized cell death mechanism through which cytotoxic agents kill tumor cells.Caspase 3 is generally recognized as a key downstream effector execution phase enzyme whose task is to dismantle key cellular infrastructure to facilitate cell death and engulfment by neighboring cells. Recently, our group discovered a surprising, counter-intuitive role for caspase 3 in cancer therapy. We found that dying tumor cells use the apoptotic process to generate potent growth-stimulating signals to stimulate the repopulation of tumors undergoing radiotherapy and chemotherapy. Surprisingly, activated caspase 3 plays key roles in the growth stimulation. Caspase 3 cleaves and activates calcium-independent phospholipase A2, which in turn facilitate the production and secretion of prostaglandin E2, which can potently stimulates growth of surviving tumor cells. Deficiency of caspase 3 either in tumor cells or in tumor stroma caused significant tumor sensitivity to radiotherapy in xenograft or mouse tumors. In human cancer patients, higher levels of activated caspase 3 in tumor tissues are correlated with significantly increased rate of recurrence and deaths. We propose the existence of a "Phoenix Rising" pathway of cell death-induced tumor repopulation in which caspase 3 plays key roles.

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MITF-driven subcompartment-specific distribution of differentially cycling tumor cells in melanoma

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Dysregulated proliferation is a cancer hallmark. We aim to uncover the cell cycle dynamics of individual melanoma cells within their complex microenvironment. We have developed a model to visualize melanoma cell cycle dynamics in real-time *in vitro* and *in vivo*. Cells transfected with the fluorescence ubiquitination cell cycle indicator (FUCCI) appear red in G1, yellow in S and green in S/G2/M with a fluorescence gap during cytokinesis. FUCCI-melanoma cells were grown as 3D-spheroids and implanted into a collagen matrix to mimic tumor architecture and microenvironment, or as xenografts in NOD/SCID mice. In 3D-spheroids, initially the ratio of red:green melanoma cells was roughly equal and distributed randomly. Within hours the interior cells became slow-moving and arrested in G1, while peripheral cells were cycling, highly motile and invaded cell cycle phase-independently the collagen matrix. We sorted and cultured the interior cells in 2D. Live-cell imaging revealed that the G1 arrested population re-entered the cell cycle, however with a lag compared to the outer population. Intravital multiphoton microscopy of FUCCI-melanoma xenografts visualizing cell motility relative to intact tumor vasculature in live mice revealed that the tumors can be divided into two groups: Xenografts derived from MITFhigh melanoma lines proliferate heterogeneously throughout the tumor, while MITFlow lines proliferate predominantly at the tumor periphery and in close proximity to capillaries, while most cells further away from oxygen and nutrient supply arrest in G1. Our data suggest that MITF expression dictates the subcompartment-specific distribution of differentially cycling tumor cells in melanoma, which may result in differential sensitivity to apoptosis and therefore may contribute to the resistance of melanoma to therapy.

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Intratumoral vaccination of Propionibacterium acnes induces Th1 immune responses and suppresses the growth of malignant melanoma

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Malignant melanoma (MM) is an aggressive cutaneous malignancy associated with poor prognosis; many putatively therapeutic agents have been administered, but with mostly unsatisfactory results. Propionibacterium acnes (P. acnes) is an aero tolerant anaerobic gram-positive bacteria that causes acne and inflammation. P. acnes taken in phagocytes induces a strong Th1-type cytokine responses producing IL-12, IFN-γ and TNF-α. Previously, we have successfully controlled experimental dermatitis in a model mouse by P. acnes vaccination correcting Th2 type cytokine milieu. In the present study, using a selected P.acnes strain that induces strong Th1 responses *in vitro*, P. acnes immunotherapy against MM was evaluated. The B16 melanoma cells were implanted in the 8 week-old mice dorsal skin. Heat-killed P. acnes were injected in the implanted site simultaneously and/or 2 weeks later. Intratumoral administration of P. acnes successfully protected the host against melanoma progression *in vivo* with histopathological disappearance of the planted melanoma cells. The vaccination induced both cutaneous and systemic Th1 type cytokine expression, including TNF-α and IFN-γ, which are associated with subcutaneous granuloma formation. The phagocytes forming a granuloma to be a long-lasting Th1 type cytokine source *in vivo*. In fact, P. acnes-treated tumor lesions were densely infiltrated with TNF-α and IFN-γ bearing T cells. In spleen, TNF-α or IFN-γ producing CD8+ T cells and monocytes number was increased. Present data showed that P. acnes vaccination induces lesional and systemic antitumor responses suggesting a potent therapeutic alternative for MM.

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The effect of 2-hexyldecanol on reducing hyperpigmentation and suppressing melanin synthesis via activation of ubiquitin-proteasome system

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2-hexyldecanol (HD) is an aliphatic alcohol that has been used as an emollient in beauty care products safely for a long time. However, no biological efficacy has been reported in the past. We investigated the effect of HD on modulating the proteasome activity and melanin synthesis in melanocyte in in-vitro models. Using a proteasome protease-specific substrate (Suc-Leu-Leu-Val-Tyr-AMC), we found HD up-regulated proteolytic activity 2-fold, and the effect was mostly counteracted by the presence of MG132, a proteasome protease-specific inhibitor, suggesting that HD specifically up-regulates proteasome activity. We then evaluated the effect of MG132 and HD on melanin synthesis in a B16 melanoma cell culture model using a spectrophotometric measurement at OD410 as an endpoint. Addition of MG132 significantly increased melanin synthesis in a dose-response manner, which implies down-regulation of degradation of melanin synthesis related proteins leading to increased melanin synthesis. Strikingly, addition of HD strongly reduced melanin synthesis in a dose-response manner, while addition of MG132 completely reversed its suppression activity, which confirmed antagonistic roles in acting on the ubiquitin-proteasome pathway. To confirm its effectiveness, we tested an oil-in-water product containing HD in a human skin explant model. After 7 days of topical treatment, the product significantly suppressed the production of melanin vs. its vehicle. Lastly, we evaluated the effect of a HD-containing formula on facial hyperpigmented spots among Chinese females in a randomized, vehicle-controlled, double-blinded clinical study. After 8 weeks of treatment, the HD-containing formula significantly reduced the appearance of facial spots vs. its vehicle ($p<0.01$). These data indicated HD is effective in improving facial hyperpigmentation, and its mechanism of action can be attributed to reducing melanin synthesis via upregulation of the ubiquitin-proteasome pathway in melanocytes.

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Oxidative stress induces translocation of calreticulin in cultured human melanocytes cells

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Oxidative stress is attributable to the apoptosis of skin cells, leading to various types of skin disorders such as vitiligo. UV radiation is recognized to be one of the most critical factors in inducing oxidative stress for skin cells including melanocytes. Calreticulin or CRT is the residential proteins on the membranes of endoplasmic reticulum or ER, and has a wide range of physiological and pathological functions. Recent studies have shown that translocation of CRT from ER to cell surface enhances immunogenic cell death. We undertook this project to investigate whether oxidative stress induces CRT translocation in cultured human skin melanocytes. Confocal microscopic data showed that H2O2 induces CRT translocation in a dose and time dependent manner in cultured human skin melanocytes. Cell membrane separation and Western blot analysis confirmed the observation. Contrary to UV-induced CRT translocation in cultured human skin keratinocytes where PERK is activated, H2O2 does not induce PERK activation in cultured human skin melanocytes. Collectively, our data suggest that UV radiation or other factors-induced oxidative stresses induce CRT translocation that enhances recognition by immune active dendritic cells and in turn potentiate the loss of melanocytes. This may add to the list of recognized causes of the pathogenesis of vitiligo. Further understanding of the mechanism of CRT translocation in melanocytes may unravel the molecular targets for intervention of vitiligo and offer better clinical approaches.

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Application of dopamine and L-Dopa-loaded chitosan nanoparticles to enhance melanin production in cultured human skin melanocytes

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Among various treatments for vitiligo is the autologous skin transplantation, which remains the most efficient clinical approach. Recent advancements in *in vitro* melanocyte cell culture and tissue engineering technology have further enhanced the efficacy and reduced the invasiveness of vitiligo treatment. A cellular patch with a layer of biomaterial membrane with cells cultured on its surface has been developed to minimize cell damage and improve engraftment efficiency. During the transplantation, patients' de-epithelized vitiliginous areas are covered with the patch's cell side. The cells will then migrate from the patch onto the wound bed and proliferate and restore pigment. We previously developed transferable cross-linked chitosan membranes for human melanocyte culture. However, melanocytes are inevitably challenged in the novel niche. The survival and the production of melanin of melanocytes remain to be studied. We undertook this project to investigate the cell survival and melanin production in melanocytes supplied with dopamine and L-Dopa-loaded chitosan nanoparticles. Melanocytes were cultured alone or co-cultured with keratinocytes, then treated with dopamine and L-Dopa, or dopamine and L-Dopa-loaded chitosan nanoparticles. We found that all treatments did not affect the cell survival and proliferation. Both dopamine and L-Dopa increase melanin production in a dose and time dependent manner, as do dopamine and L-Dopa loaded chitosan nanoparticles. While dopamine and L-Dopa are expectedly oxidized over time, dopamine and L-Dopa-loaded nanoparticles sustain oxidation even two weeks after preparation. This adds to the advantages of chitosan nanoparticles. Taken all those together, our data suggest that dopamine and L-Dopa loaded nanoparticles could be applied for the enhancement of melanin production in melanocytes used in skin transplant for vitiligo patients.

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Human melanoma cells resist to oxidative stress due to active EGFR/AKT and overproduction of melanin

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Melanoma is considered as one of the most aggressive and less treatable cancers. While various models of mechanisms have been proposed, the transformation from quiet melanocytes to invasive melanoma cells remains an enigma. Alteration of cellular redox status has been suggested to be associated with cellular transformation. We compared primary human melanocytes and melanoma cells and their responses to oxidative stress. Both cells were treated with H2O2 at doses between 5 and 250μM. Microscopic data showed that melanoma cells are more resistant to H2O2 treatment. Western blot and confocal microscopic data showed that in melanoma cells, H2O2 does not induce CRT translocation which is considered as a mechanism through which melanocytes surrender to oxidative stress and invite immunogenic cell death. Furthermore, Western blot analysis showed that melanoma cells express EGFR, responsive to EGF treatment and have constitutive activation of AKT and mTOR, which are all related to better survival. In contrast, in the primary human melanocytes, EGFR expression is almost undetectable. And confocal microscopic data confirm this observation. We also observed that in melanoma cells, but not in melanocytes, low dose (5 to 250μM) of H2O2 induces melanin production that is recognized to protect from further oxidative stress. Collectively, our data suggest that active EGFR/AKT/mTOR and up-regulation of melanin production contribute to the resistance of melanoma cells to oxidative stress and attribute to the aggressiveness of melanoma. Our data provides insights into the understanding of the molecular mechanisms of the transformation from melanocytes to melanoma and support the notion from the most recently published proteomic studies that EGFR remains one of the most important targets for better clinical management of melanoma.

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Reversal of the melanoma malignant phenotype by an endogenous cyclic peptide which mediates contact inhibition of growth

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The aim of this study was to identify a molecule believed responsible for initiating reversal of the melanoma malignant phenotype, a capability first found in tissue culture medium from a revertant line of hamster melanoma cells that contained a soluble "contact inhibitory factor" (CIF) which restored contact-, serum-, and anchorage-dependent growth control to melanoma cells and other cancers. Growth arrest was at the physiologic early G1 checkpoint. CIF increased susceptibility to both humoral and cell-mediated immune surveillance by (1) inducing synthesis of vitiligo-related pigment differentiation antigens (a proven target for ADCC and complement-mediated lysis), and (2) upregulating class I and II MHC, increasing lysis by cytotoxic T cells. In immunocompetent animals *in situ* administration of an ethanol precipitate or protein extract of CIF led to permanent regression, respectively, of 100% of hamster melanomas (6/6 vs 0/18 controls) and 3 of 4 mouse Lewis lung carcinomas; given ip to nude mice the protein extract caused sustained inhibition but not elimination of human melanomas. CIF containing culture medium suppressed FGF2-induced angiogenesis in the mouse cornea model, secretion of VEGF by melanoma cells *in vitro*, and melanoma metastasis in a chick embryo model. It also inhibited mTOR signaling in human breast and mouse brain cancer cells. We now describe bioassay- guided purification of CIF activity by column chromatography followed by mass spectroscopy, revealing a hitherto overlooked low MW cyclic peptide dimer which restores contact inhibition of growth to melanoma cells. Restoration of contact inhibition by CIF-containing medium or material derived from it was strictly correlated with, and an obligatory prerequisite for, all of the other "factor"-induced phenotypic changes described. The CIF cyclic peptide is the first molecule which reverses the melanoma malignant phenotype and supports the concept of phenotypic reversion as a valid epigenetic approach to control of melanoma and other cancers.

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Immunohistochemical evaluation of molecules that mediate the basement membrane adhesion for normal and abnormal melanocytes

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Melanocytes are thought to reside in the basal layer of the epidermis and attach to keratinocytes and the basement membrane (BM). Molecular structures of the BM underneath keratinocytes are well studied, but little is known about how melanocytes in normal skin interact with the epidermal BM. In order to better characterize the interaction between melanocytes and the BM, we performed confocal microscopic analyses on normal skin tissue in combination with melanocyte marker MelanA/Mart-1 and known molecules that are implicated in the BM adhesion by keratinocytes. MelanA positive melanocytes were localized underneath CK5/14 positive basal keratinocytes. Unexpectedly, a majority of melanocytes positioned within or below collagen IV, a major protein component of the lamina densa of the BM, rather than above it. In addition, collagen IV staining wrapped around melanocytes which was particularly unique. Integrin β1, laminin511, and CD151 were co-stained with MelanA, while integrin β4, laminin332, and collagen XVII were not. We extended our staining examinations to nevus and melanoma cases. Collagen IV stained the nevus cells in the dermis, whereas staining of integrin β1 was clearly reduced in the tumor cells. Most strikingly, the Pagetoid cells in the epidermis of melanoma tissue were not stained with integrin β1. This suggests a fundamental importance of integrin β1 to adhere melanocyte lineage cells to collagen IV, and that loss of integrin β1 might be the first key change in melanoma cells to detach from the BM. Our results revisit the questions of how to define a BM and whether melanocytes have a specialized adhesive structure. Given that melanoma, a malignant neoplasm of melanocytes, eventually detaches from the BM, and migrates to the upper epidermis or invades the dermis, our findings will help provide a better picture of the molecules involved in invasion of melanoma.

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Mutation analysis of pre-malignant and malignant pigmented lesions in xeroderma pigmentosum: The role of UV damage in melanoma induction

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Pre-malignant pigmented lesions are considered to be precursors of melanomas. In order to study melanoma evolution, we are examining early mutagenic changes in pre-malignant pigmented skin lesions, melanomas *in situ* and invasive melanomas in xeroderma pigmentosum (XP) patients. XP is a rare autosomal recessive disease, with sun sensitivity and 10,000-fold increase in skin cancers including melanomas. XP patients have defective post-UV DNA nucleotide excision repair that leads to accumulations of DNA mutations. We reported previously that mutations in the PTEN tumor suppressor gene were present in 56% of 59 XP melanomas and 91% of the 54 mutations were UV type (PNAS 106:6279 (2009)). We now examined 16 pre-malignant lesions (benign junctional nevi, lentiginous junctional nevi, and junctional nevi with atypia) from 4 XP patients who had biopsies at the NIH Clinical Center. Using laser capture microdissection, we obtained tissue samples with histologic features consistent with either premalignant pigmented lesions or melanoma. We then performed direct sequencing of DNA from these lesions and found that 50 % of the pre-malignant pigmented lesions had PTEN mutations, of which 69% were UV type. Of 13 PTEN mutations only 2 were present in both the pre-malignant lesions and the melanomas. Interestingly, the non-UV type mutation BRAF V600E and the UV-type NRAS codon 61 mutations often found in non-XP melanomas, were not present in the pre-malignant pigmented lesions (0/9, 0/8), or the melanomas (0/53, 0/51) from the XP patients. This study provides strong support for the role of UV in induction of PTEN suppressor gene mutations in pre-malignant and malignant pigmented lesions in XP patients. However, only a small proportion of the mutations in the pre-malignant lesions were associated with malignant degeneration. BRAF and NRAS oncogene activation appears to be of less importance in the XP melanomas.

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A locked, dimeric CXCL12 variant effectively inhibits pulmonary metastasis of CXCR4-expressing melanoma cells due to enhanced serum stability

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We previously demonstrated that the CXC chemokine receptor-4 (CXCR4) enhances the pulmonary metastasis of B16 melanoma cells ten-fold following IV inoculation. We have also reported that wild-type CXCL12 (wtCXCL12) can inhibit metastasis *in vivo* under the hypothesis that wtCXCL12 dimerizes at high concentration to become a potent antagonist of CXCR4 (in contrast to monomeric CXCL12 which acts as an agonist). To confirm this hypothesis, we genetically engineered a covalently locked dimeric variant of CXCL12 ($CXCL12_2$) and produced it in milligram quantities in E. coli for *in vivo* studies and for *in vitro* analysis of its stability in serum-containing medium. The locked $CXCL12_2$ variant inhibited wt CXCL12-induced chemomigration at an IC_{50} of 19 nM. Herein, we show that $CXCL12_2$ inhibits lung metastasis of CXCR4-B16-F10 cells more effectively than the same concentration of wtCXCL12 or AMD3100. To determine a mechanism for the enhanced antagonistic properties of $CXCL12_2$, we performed western blot and ELISA analyses, which revealed that $CXCL12_2$ was stable for up to 12h incubation in the presence of serum whereas wtCXCL12 was quickly degraded. $CXCL12_2$ also maintained its antagonist properties in chemotaxis assays for up to 24h incubation with serum, whereas wtCXCL12 was ineffective after 6h. Heat-inactivation of serum at 56 deg. C prolonged the stability and function of wtCXCL12 by more than 6 hours, suggesting enzymatic degradation as a possible mechanism for wtCXCL12 inactivation. Incubation of small ubiquitin-related modifier (SUMO)-tagged wtCXCL12 with serum resulted in proteolysis at the CXCL12 N-terminus, confirming the susceptibility of wtCXCL12 to degradation. In summary, our results suggest $CXCL12_2$ possesses greater potential as an anti-metastatic drug compared to AMD3100 and that it more effectively inhibits CXCR4-expressing melanoma lung metastasis compared to wtCXCL12 because of its enhanced serum stability in the presence of N-terminal degrading enzymes.

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Novel gene expression changes in the microenvironment of vitiligo lesional skin

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Vitiligo lesions are characterized by the death of melanocytes. However, the mechanism of pathogenesis remains unclear. It has been speculated that vitiligo lesional microenvironment is toxic or non-favorable to the growth and survival of the melanocytes. However, convincing evidence has been lacking. In this study, we performed transcriptome analysis of vitiligo lesional skin (N=17) in comparison with the perilesional normal appearing skin (N=17) from vitiligo patients and health skin from unaffected individuals (N=9). Of the 41,000 unique human transcripts screened, 63 showed significant differential expression (>2 fold, $p<0.05$ after Bonferroni correction) between lesional and non-lesional skin. Three groups of gene changes can be recognized: (1) melanocyte markers, which were dramatically decreased in lesional skin; (2) inflammation markers involved in innate and adaptive immunity, which were increased in lesional skin; and (3) keratinocyte-expressed genes, a C-type lectin expressed by the basal keratinocytes. This lectin, which is an inhibitor of the innate and adaptive immunity, was reduced by more than 8 folds in vitiligo lesional skin. Based on these observations, we postulate that the microenvironment, including the keratinocytes, contain previously unknown molecular changes that may contribute to vitiligo pathogenesis. Future functional characterization is needed to test if these changes are the cause or effect of melanocyte death.

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A unique three-compound association to improve melasma and solar lentigo hyperpigmentation

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Melanin is a biopigment, synthesized within melanocytes. These cells are localized in the basal layer of the epidermis. They transfer melanin to surrounding keratinocytes. The quantity, type and distribution of melanin in keratinocytes are one determinant of human skin color. Although melanin plays a critical role to protect skin from solar UV radiation, excess melanin synthesis can lead to hyperpigmentation disorders such as melasma, lentigo and age spots. Therefore, development of whitening products is of great interest to improve clinical and cosmetic concerns. Our Laboratory has developed *in vitro* assay to screen inhibitors of melanin synthesis. We have found that delta tocopheryl glucoside strongly inhibits melanin synthesis in B16-F10 cell line. Besides, the association of delta tocopheryl glucoside with another compound, called melanyde, leads to a synergic action in this model. We have shown that this latter molecule is a strong inhibitor of human tyrosinase. Both compounds, Delta tocopheryl glucoside and melanyde were associated with retinaldehyde in a cosmetic composition. It was tested on a pigmented reconstructed epidermis (melanoderm, Mattek). The results showed a significant visual whitening effect after repeated topical applications. Finally, two clinical studies were conducted to assess the efficacy of the three-compound association. First study was led on 60 subjects with pigmented spots (lentigos) on the back of the hands. Second study was led on 90 subjects with melasma. The statistical analysis of the results showed a significant whitening effect at the end of treatment with regard to the beginning of studies in both cases. To conclude, we have shown that the unique association of delta tocopheryl glucoside, melanyde and retinaldehyde had a significant whitening effect and can be used to improve lentigo and melasma.

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Site-specific heterogeneity of melanocytes during late fetal periods

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A growing body of evidence indicates that melanocytes, melanoblasts, and melanocyte stem cells are closely related to hair follicles. Melanocyte stem cells exist in the hair follicle bulge region. Little is known about melanocytes in glabrous sites, such as the palms and soles. Here we investigated whether the melanocytes of the sole differ from those at other sites by obtaining skin specimens and isolating melanocytes from the skin at 4 different body sites (scalp, back, abdomen, and sole) of a human fetus. Immunohistochemical studies of biopsy tissues obtained from 31 aborted fetuses (from 13-21 weeks gestation [WG]) revealed that fetal sole melanocytes localize only in the apertural area of eccrine sweat gland ducts. Immunocytochemical studies using cultured cells revealed that these cells were melanoblasts or melanocyte precursors. Gene expression studies, including microarray analysis, Western blotting, and real-time polymerase chain reaction using cultured melanocytic lineage cells derived from 3 different fetuses (13WG, 17WG, and 19WG) indicated that the gene expression profiles of fetal sole melanocytic lineage cells were different from those of other melanocytic lineage cells derived from hairy sites (scalp, back, and abdomen). Expression of some genes, including TBX4, DKK1, WIF1, FGF7, and CHI3L1, was significantly higher in the sole cells. Melanogenic genes, including TYRP1, DCT, SILV(GP100), and MLANA(MART-1), were expressed in only low levels in the sole cells. In particular, high expression of FGF7 and DKK1 was observed only after completion of eccrine sweat gland formation. The maturation process, related to eccrine sweat glands and not to hair follicles, may account for the difference in sole melanocytes.

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Melanoma simulation model: Promoting opportunistic melanoma screening and appropriate patient counseling through education

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The simulation model ensures that every medical student has the opportunity to learn assessment criteria for pigmented lesions to prepare them to recognize melanoma during physical examinations. Two simulation models replicated clinically suspicious pigmented lesions with known pathology. Dermatologists assessed the lesions for reliability, and lesions failing to achieve inter-rater agreement were replaced. During the monthly rotation of the primary care clerkship, third year medical students participated in a session demonstrating a 3 integer scoring system for visual assessment of border, color and diameter of the lesion. The sum of the 3 scores defined threshold rules for clinical management of the lesion. Four small groups of 3-4 students first assessed Model 1 using the criteria, presented their findings and discussed patient counseling. Two weeks later, the students assessed Model 2. The main outcome measure was interobserver agreement on scoring lesions by 70 students. A secondary outcome was application of the threshold rules to appropriately manage the patient. The overall agreement for 17 lesions consisting of 6 melanomas, 1 melanoma *in situ* and 10 benign was 77% for diameter, 58% for color, and 46% for border. For benign lesions, 49% of students utilized the threshold rule to decide to reassure the patient, 29% to re-check in 3 months, and 21% referred to a dermatologist for assessment. Most students (59%) referred to a dermatologist for assessment of melanoma *in situ*, 29% re-checked in 3 months, and 3% referred for biopsy. For melanomas, 34% referred for a biopsy, 47% referred to a dermatologist for assessment, and 14% watched for change in 3 months. Even though students had difficulty assigning scores to the border and color, the threshold rules supported the correct decision for patient management of melanoma (81%) and melanoma *in situ* (91%).

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Chronic downregulation of the Sema4D receptor, Plexin B1, results in c-Met activation in melanocytes

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Plexin B1 is a type I transmembrane receptor for Semaphorin 4D (Sema4D), and is expressed in human melanocytes and lost in melanoma *in vivo* and *in vitro*. We have shown that Plexin B1 is a tumor suppressor protein for melanoma, in part through its ability to suppress activation of the oncogenic c-Met receptor by its ligand, hepatocyte growth factor (HGF). In this report we examined the effect of chronic down-regulation of Plexin B1 in melanocytes on the activity of the c-Met receptor. Silencing of Plexin B1 using Lentiviral vectors in melanocytes cultured from two individuals resulted in activation of c-Met, in the absence of ligand. Gab1, an adaptor protein for c-Met, was activated in Plexin B1 silenced cells in a c-Met-dependent manner, as shown by experiments using a specific c-Met inhibitor. Migration of Plexin B1 silenced cells was significantly increased compared with shRNA controls, and was c-Met dependent. Plexin B1 silenced cells showed downregulation of the cell-cell adhesion molecule E-cadherin, a c-Met target. Constitutive activation of c-Met was not due to de novo production of HGF by melanocytes, as demonstrated by Western blotting of Plexin B1 silenced cells for HGF, and by use of blocking HGF antibodies, which failed to suppress c-Met activation in Plexin B1 silenced cells. To determine the mechanisms by which loss of Plexin B1 activates c-Met, we determined if Plexin B1 and c-Met exist in a complex, using Imagestream analysis. Imagestream is a FACS technique for assessment of individual cells, and can identify and quantitate receptor co-localization. Preliminary results suggest that c-Met and Plexin B1 exist in a complex in normal human melanocytes, which may suppress basal levels of c-Met oligomerization and activation. These results are important because they identify a co-receptor for c-Met in normal human melanocytes that regulates its activation. Because Plexin B1 is down regulated by UVB in melanocytes, UVB may regulate c-Met-dependent functions in melanocytes indirectly through effects on Plexin B1 expression.

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MC1R determines melanocyte viability upon UV irradiation by protecting PTEN activity

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The melanocortin-1-receptor (MC1R) is a trimeric G protein coupled receptor that is activated by α-melanocyte-stimulating hormone (α-MSH). The activation of this signaling pathway leads to an increase in intracellular cAMP, which is responsible for constitutive pigment variation in humans. Here, we provide *in vitro* and *in vivo* evidence that beyond its previously known role in stimulating melanogenesis, MC1R signaling also regulates melanocyte viability through modulation of PTEN activity. Specifically, MC1R signaling regulates melanocyte proliferation, and impairs the ability of melanocytes to repair DNA and ROS production in response to damages caused by UV irradiation due to interaction with PTEN protein in a p53-independent manner. MC1R protein binds with PTEN protein to protect PTEN activity, and eventually to inhibit AKT phosphorylation and PTEN oxidation after UVB irradiation. Some variants of MC1R (e.g. R151C, R160W, and D294H) which have high association with melanoma incidence in human dysfunctions in the regulation of melanocyte proliferation, ROS production and DNA repair due to abnormal interaction with PTEN. Given the vital roles of MC1R variants in DNA repair and melanoma development, these studies establish PTEN as a central interactor of MC1R in UVB response in melanocytes.

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Differential roles of IL-1α and IL-1β in tumor progression

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While the IL-1 superfamily of cytokines and receptors has expanded greatly in the past 5 years, the biological roles of the original family members remain incompletely characterized. There are two forms of biologically active IL-1: IL-1α and IL-1β. IL-1α is constitutively expressed by epithelial cells, while IL-1β is predominantly produced by activated immune cells. All known biological activities of IL-1α and IL-1β are mediated by their binding to IL-1 receptor type 1 (IL-1R1). All these elements of the IL-1 are represented abundantly in skin. The relative roles of these IL-1 elements in skin tumor progression are controversial. In this study, we injected intradermally the EL4 T lymphoma cell line in the skin of transgenic mice deficient in either IL-1α, IL-1β or IL-1R1. We observed that while IL-1α and IL-1R1 deficiencies do not affect tumor progression, that IL-1β deficient mice showed markedly reduced tumor growth. Similar results were obtained with the B16 melanoma cell line. Because IL-1R-/- mice can not transduce signals from either IL-1α or IL-1β, and accommodate normal tumor progression, we conclude that it is both the absence of IL-1β and the presence of IL-1α that are responsible for the markedly reduced tumor progression in IL-1β -/- mice. This reduced tumor progression in IL-1β-/- mice is dependant upon T cell mediated immunity, because depletion of T cells from IL-1β-/- mice completely restored rapid tumor growth in these mice. Interestingly, normal BM→IL-1β-/- chimeric mice, but not IL-1β -/-BM→normal chimeric mice, reproduced the reduced tumor growth seen in IL-1β-/- mice, suggesting that IL-1β deficiency in skin stromal cells is involved in the suppression of tumor growth. We conclude from that IL-1α and IL-1β play distinct roles in skin tumor growth: IL-1α is required for an optimal anti-tumor immune response, while IL-1β can promote tumor progression. This work suggests that IL-1β blockade may have a role in anticancer therapy.

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Chimeric 9p-22q transcript in a patient with melanoma and DNA repair deficiency acts as a negative regulator of p14ARF

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The incidence of melanoma is increasing and appears to be associated with mutations in several critical genes including the cell cycle regulator CDKN2A. The regulation of p14ARF encoded by CDKN2A gene in melanoma remains largely unexplored. Here, a novel fusion transcript of p14ARF to a new gene in a gap on 22q11.2 derived from a novel t (9; 22) translocation was detected in a 12 y/o patient with melanoma, DNA repair deficiency, congenital deafness and features suggestive of DiGeorge Syndrome. Chromosome sorting, arrayCGH and PCR localized the 9p21 breakpoint to p14ARF intron 1 and the 22q11.2 breakpoint to a palindrome AT rich repeat (PATRR) in an unsequenced gap. p14ARF was under-expressed and the DNA repair deficiency was corrected by transfection with p14ARF. Laser capture microdissection of his melanoma revealed additional UV–type p14ARF mutations implicating p14ARF in his melanoma. There were no significant differences in expression of p53, mdm2, p21, p16INK4a or XPC in this patient compared to cells from his parents and normal controls. Reduced p14ARF expression was not elevated in patient cells after exposure to UV, suggesting that p14ARF may directly involve DNA repair by an ARF-p53-Mdm2 pathway and XPC-independent mechanism. A chimeric transcript (p14ARF-PCTH) was detected in the patient cells. Inhibition of p14ARF-PCTH by siRNA resulted in increased levels of p14ARF mRNA and protein and improved DNA repair capability. Since the chimeric transcript is involved in regulation of p14ARFf expression it could provide a unique target for skin cancer prevention in this patient.

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A cosmetic treatment that contains ingredients that target melanogenesis evens skin tone and reduces both discrete and mottled pigmentation *in vivo*

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Topical cosmetic treatments that treat unwanted pigmentation are highly sought after especially in Asia. These cosmetic formulations contain technologies that reduce pigment production in skin. Tyrosinase inhibitors such as kojic acid, and licorice extract are commonly used. As there are multiple biological pathways involved in pigment formation, new approaches have been utilized to enhance the ability of cosmetic treatments to treat unwanted pigmentation. We identified several key ingredients that inhibit pigment formation through different biological pathways. We developed a cosmetic treatment using a combination of technologies that inhibit gene promoters of key pigmentation genes such as MITF, and TRP-1, inhibit tyrosinase enzyme activity, as well as inhibit melanocyte pigment formation. Clinical tests using this cosmetic treatment was found to reduce discrete and mottled pigmentation and improve overall skin tone.

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Vitiligo inducing phenols increase production of IL6 and IL8 from melanocytes via the unfolded protein response

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Vitiligo is characterized by depigmented skin patches due to loss of melanocytes. Etiology of vitiligo is not fully understood but oxidative stress is thought to play an early role in vitiligo, while autoimmunity contributes to disease progression. In this study we sought to identify mechanisms that link disease triggers and spreading of lesions. A hallmark of melanocytes at the periphery of vitiligo lesions is dilation of the endoplasmic reticulum (ER). We hypothesized that redox disruptions extend to the ER where oxidation/reduction reactions facilitate protein folding and the subsequent accumulation of misfolded peptides activate the unfolded protein response (UPR). We used 4-tertiary butyl phenol (4-TBP) and monobenzyl ether of hydroquinone (MBEH), known triggers of vitiligo, to study pathways that contribute to melanocyte loss. Expression of key UPR components including IRE1 and PERK was increased following exposure of human melanocytes to the phenols. PERK activation led to enhancement of the antioxidant response by recruitment of the transcription factor NRF2 to the nucleus and increased expression of the antioxidant HMOX1. Splicing of the transcription factor X-box binding protein-1 (XBP1) was increased following exposure of melanocytes to phenols. XBP1 activation increased production of interleukin-6 (IL6) and IL8. XBP1 inhibitors reduced IL6 and IL8 production induced by phenols, while over-expression of XBP1 alone increased their expression. Thus, in response to chemicals known to cause vitiligo, melanocytes themselves produce cytokines associated with activation of an immune response. Increased levels of inflammatory cytokines have been observed in serum and tissue from vitiligo patients. IL6 and IL8, in particular, are expressed at the periphery of vitiligo lesions and may contribute to recruitment of immune components, perpetuating melanocyte loss. These results expand our understanding of the mechanisms underlying melanocyte loss in vitiligo and pathways linking environmental stressors and autoimmunity.

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DC-HIL$^+$ myeloid-derived suppressor cells (MDSC) are a new prognostic marker for melanoma and promising target for immunotherapy

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MDSC are the most potent suppressors of T-cell function and their exponential proliferation during cancer progression severely restricts efficacy of immunotherapy. Having discovered DC-HIL as the surface receptor responsible for the immuno-suppressive activity of MDSC generated by melanoma in mice, we posit that DC-HIL expression by MDSC may be a useful parameter in managing melanoma patients. By FACS, we found CD14$^+$HLA-DR$^{neg/low}$ MDSC from peripheral blood cells (PBC) to be significantly different (p=0.03) in patients with metastatic (Stage IV) melanoma (n=16) (2.0 ± 1.2%) vs. healthy donors (n=7) (0.4 ± 0.6%). We next examined DC-HIL$^+$ MDSC relative to cancer stage. DC-HIL was expressed by almost all MDSC (97 ± 43%) from patients with metastasis (Stage III-IV) (n=14), but reduced significantly (64 ± 38%) (p=0.02) in patients with thin (<1 mm) melanoma (Stage 0-IA) (n=11). By contrast, only 17 ± 10% of MDSC from patients with atypical nevi (n=3) and just 8 ± 7% of MDSC from healthy donors (n=7) were DC-HIL$^+$. We sorted cases of thin melanomas into thicker than *in situ* vs. *in situ* tumors, which revealed divergent DC-HIL$^+$ fractions (84 ± 19% vs. 54 ± 38%). Having shown anti-DC-HIL Ab to abrogate MDSC's immunosuppression in mice, we examined this Ab's ability to rescue CD8$^+$ T-cell function suppressed by melanoma in humans. PBC from Stage III-IV patients were treated with anti-DC-HIL (or control) Ab, and then assayed for IFNγ secretion. DC-HIL blockade led to 4-fold rise in IFNγ, associated inversely with IFNγ level in untreated cultures (r = -0.7). Our results show strong positive correlation between DC-HIL$^+$ MDSC and cancer staging, and the great potential for boosting CD8$^+$ T-cell activity by blocking DC-HIL function in melanoma patients. Thus, blood DC-HIL$^+$ MDSC are a useful marker of melanoma progression that may refine prognostication, including stratification of thin melanomas. DC-HIL antagonists offer hope for improved melanoma treatment.

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BI 6727, a second generation small molecule inhibitor of polo-like kinase 1 (Plk1), causes significant melanoma growth delay and regression *in vivo*

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Melanoma remains one of the most aggressive skin cancers, which is potentially lethal, if not diagnosed and treated early. According to estimates from the American Cancer Society, melanoma incidence rates have increased among white population during 1999-2008. Further, 76,250 new cases of melanoma are expected to be diagnosed in 2012 and 9,180 deaths are expected due to this cancer. Therefore, concerted mechanism-based new efforts are needed to combat this neoplasm. In this regard, a better understanding of the genetic control of cellular proliferation and cell division may be useful towards rational designing of specific therapeutics for melanoma management. We have earlier shown that the mitotic serine/threonine kinase Plk1 is over-expressed in human melanoma and its inhibition causes mitotic catastrophe and induction of apoptosis in human melanoma cells. In this study, we determine the anti-proliferative efficacy of BI 6727, a second generation Plk1 specific small molecule inhibitor, *in vivo* in athymic nu/nu nude mice implanted with human melanoma cells. For this study, A375 cells ($1x10^6$ cells) were implanted in athymic nude by subcutaneous injection on the right flanks. The tumors were allowed to grow and the mice bearing established tumors (>100 mm^3) were randomized into 3 groups, each containing 12 mice. The animals were treated with BI 6727 (10 mg/kg or 25 mg/kg; *intravenous injection*) or vehicle, twice weekly. We found that BI 6727 treatment resulted in a significant delay in tumor growth (time to reach to a 1000 mm^3 target volume). Interestingly, higher dose of BI 6727 (25 mg/kg) resulted in a near complete tumor regression, which was stable for several days after treatment discontinuation. In addition, we found that BI 6727 caused i) a significant inhibition in cellular growth/viability, and ii) induction of apoptosis in multiple melanoma cell lines. Based on this study, we suggest that Plk1 is a potential druggable target for melanoma management.

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A genome-wide shRNA screen identified the WNT pathway as a potential target for combination treatments with BRAF inhibitors in melanoma

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Approval of PLX4032, an inhibitor of BRAFV600E, for treating melanoma is exciting due to the positive response observed in patients. However, many patients with this single-agent treatment relapse eventually. Thus, combination treatments will be required to overcome this resistance. To identify druggable targets to be combined with this or other BRAF inhibitors, we screened for genes and pathways whose inhibition increases the efficacy of this treatment on melanoma cells using a large scale RNAi-based synthetic lethal screen with a BRAF inhibitor, PLX4720 (structurally similar to PLX4032). We used a lentiviral genome-wide human short-hairpin RNA (shRNA) library and Illumina Genome AnalyzerIIx deep-sequencer. With our BiNGS! Analysis System, we identified 744 genes and several pathways that could sensitize cells to this BRAF inhibitor when down-regulated. Some of the pathways identified include the PDGF, PI3K, and MAPK signaling pathways, all of which have been reported to contribute to melanoma resistance to BRAF inhibitors. Experiments using individual shRNAs targeting genes in the top gene list have validated our screen for seven out of nine top genes, including TCF3. Furthermore, knockdown of multiple genes involved in the WNT pathway seem to increase the sensitivity of cells to PLX4720, suggesting that the WNT pathway is a potential target for combination treatments against melanoma. Further validation experiments with compounds targeting WNT pathways are on-going. In conclusion, the results here validated our approach for identifying potential druggable targets for combination treatment of melanoma with BRAF inhibitors, and the WNT pathway is one of them.

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Strong down-regulation of miR-26a in melanoma and therapeutic potential

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Increasing evidence points to the importance of microRNAs (miRNA) in the development of cancer. Melanoma is an aggressive cancer that metastasizes rapidly, and is refractory to conventional chemotherapies. Identifying miRNAs that are responsible for this pathogenesis is therefore a promising means of developing new therapies. In an initial microarray screen, we identified miR-26a as an miRNA that is down-regulated in melanoma cell lines as compared to primary melanocytes. Subsequent validation by qPCR showed that miR-26a is strongly down-regulated in melanoma cell lines by roughly 17-fold on average compared to melanocytes. Treatment of melanoma cell lines with miR26a mimic caused significant and rapid cell death compared to scrambled control in most cell lines tested. In testing a number of potential targets of miR-26a, we found that SMAD1 and SODD (BAG-4) were both significantly reduced when cells were treated with miR-26a mimic in those cell lines that were sensitive. Further validation experiments to examine whether the effectiveness of miR-26a mimic is mediated primarily through these two proteins are ongoing. Our results indicate that miR-26a down-regulation is important for the survival of melanoma cells, and that replacement of miR-26a in melanoma patients, or the direct targeting of SMAD1 and/or SODD, is a potential therapeutic strategy.

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Kinetics and computational docking studies on the inhibition of tyrosinase induced by oxymatrine

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A combination of enzymatic inhibition kinetics and computational prediction were employed for searching the effective inhibitor of tyrosinase. We found that oxymatrine significantly inhibited tyrosinase, and this was not accompanied by detectable conformational changes. Kinetic analysis showed that oxymatrine reversibly inhibited tyrosinase in a mixed-type manner. Measurements of intrinsic and ANS-binding fluorescences showed that oxymatrine did not induce the conspicuous changes in the tertiary structure. We further conducted the docking simulation between tyrosinase and oxymatrine by using two kinds of docking programs such as Dock6.3 and AutoDock4.2 (binding energies were -118.81 kcal/mol for Dock6 and -8.04 kcal/mol for AutoDock4). The results also suggested that oxymatrine interacts mostly with the residues of CYS83 and HIS263 in the active site of tyrosinase. As suggested here for oxymatrine, the present strategy of predicting tyrosinase inhibition by simulation of docking coupling with kinetics may prove useful in screening for potential tyrosinase inhibitors. The knowledge of tyrosinase inhibition can provide medical, cosmetic, and agricultural applications, hence, our study suggest that oxymatrine is the applicable agent for the various applications related with pigment formations.

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Soluble adenylyl cyclase is a novel regulator of melanosomes in human melanocytes

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Skin pigmentation disorders affect over 1% of the world's population and produce impactful psychosocial and cosmetic effects on those afflicted. In addition, pigment production in melanoma helps this cancer evade chemotherapies. While much is known about the synthesis of melanin in human melanocytes, important details about its cellular regulation, degradation, and distribution to surrounding keratinocytes is yet to be explored. Within melanocytes, cyclic AMP (cAMP) is a key signaling molecule for the transcription of numerous enzymes involved in melanogenesis. We study a novel source of cAMP in melanocytes called soluble adenylyl cyclase (sAC). sAC, unlike the more widely studied transmembrane adenylyl cyclases (tmACs), is not permanently linked to the plasma membrane but localized to the nucleus, cytoplasm and mitochondria. In addition, sAC is regulated by changes in cellular pH and is known to control the trafficking of the vacuolar-type H+-ATPase (V-ATPase), a protein essential for the acidification and maturation of organelles such as the melanosome and lysosome. Pharmacological or genetic inhibition of sAC increased melanin content per unit cell. These results are intriguing because cAMP elevation, rather than inhibition, has been well characterized as an inducer of melanogenesis. Electron microscopic studies following sAC inhibition demonstrated both an elevation in total melanosome number and the accumulation of large atypical double membrane enclosed structures containing melanosomes and free melanin. These large structures are consistent with autophagolysosomes, and our lab has recently shown in neurons that sAC is essential for normal autophagy. Therefore, we hypothesize that sAC inhibition leads to elevation in melanin and melanosomes by preventing the normal process of melanosome degradation. Our results suggest that sAC is a novel regulator of melanosomes, and that further investigation of sAC may offer new mechanisms to ultimately control human skin pigmentation.

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Soluble adenylyl cyclase is essential for stem cell factor induced proliferation and Ras signaling in melanocytes

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Stem cell factor (SCF) is an essential growth factor for melanocytes controlling melanocyte development, migration, and growth. Activating mutations in the receptor for SCF, ckit, are implicated in the development of melanoma. SCF regulates melanocytes mainly through the activation of the MAPK signaling pathway by stimulating Ras. cAMP signaling pathways have long been known to alter the activity of MAPK signaling proteins such as Ras and Raf, but in many cases the source of cAMP has been poorly understood. We study a novel source of cAMP in mammalian cells called soluble adenylyl cyclase (sAC). Unlike transmembrane adenylyl cyclases (tmACs), sAC is not responsive to G proteins, and instead is regulated by calcium, bicarbonate ions, and ATP. In addition, sAC is present in the nucleus, mitochondria, and cytoplasm. We recently published that sAC is distinctly localized in benign melanocytes and melanoma. In benign melanocytes the protein is present in the cytoplasm and is enriched in a microdomain outside the nucleus while in melanoma the protein is diffusely cytoplasmic and enriched in the nucleus. Distinct localization of sAC in benign versus malignant melanocytes suggested this signaling protein may have an important function in melanocyte biology. We have found that in primary human melanocytes SCF leads to a potent stimulation in cAMP in a sAC-dependent manner. Furthermore, using both chemical and genetic sAC inhibitors, we have demonstrated that SCF-dependent Ras, B-Raf, MEK, and ERK activation require sAC activity, and that sAC activity is essential for SCF-induced melanocyte proliferation *in vitro*. These data establish a new signaling cascade downstream of SCF/ckit with novel implications for both normal melanocyte biology and melanomagenesis.

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Soluble adenylyl cyclase is a novel regulator of Ras signaling in melanoma

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The Ras/Raf/ERK signaling cascade is implicated in multiple aspects of melanoma, including melanomagenesis, invasion, and metastasis. The cAMP pathway is a known modulator of the Ras pathway, and while a key role for cAMP in melanoma pathogenesis is established, pinpointing the source of cAMP has remained unclear. We study a distinct source of cAMP in mammalian cells called soluble adenylyl cyclase (sAC). Unlike transmembrane adenylyl cyclases (tmACs), sAC is not responsive to G proteins, and instead is regulated by calcium, bicarbonate ions, and ATP. In addition, sAC is present in the nucleus, mitochondria, and cytoplasm. We have previously examined the localization of cAMP signaling microdomains in melanocytes. We recently published that in biopsied benign nevi sAC is localized within the cytoplasm with specific enrichment at the golgi, while in melanoma sAC expression is increased and protein localization is diffusely cytoplasmic and enriched in the nucleus. This distinct localization of sAC proved to be 92% sensitive and 88% specific for the diagnosis of melanoma and suggested that sAC may have a key role in melanoma signaling. We now examine the expression level of sAC in human melanoma cell lines with distinct Ras pathway mutations, and, using sAC-specific inhibitors, evaluate the role of sAC-dependent cAMP in Ras signaling. We now demonstrate that inhibition of sAC leads to a large decrease in cAMP in human melanoma cell lines. Furthermore, chemical or genetic inhibition of sAC leads to an activation of Ras, C-Raf, MEK, and ERK signaling. Activation of Ras has been previously shown to be important for the development of PLX4032 resistance in B-RafV600E melanomas, and in accordance with these observations, inhibition of sAC renders B-RafV600E melanomas insensitive to PLX4032. Soluble adenylyl cyclase represents a possible diagnostic adjunct for melanoma, and sAC-dependent cAMP appears essential for Ras-dependent signaling in melanoma.

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Live imaging analysis of melanosome transfer using lipophilic tracer

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Melanosome transfer is an important biological activity for skin pigmentation. The mechanism remains controversial. In order to elucidate the mechanism of melanosome transfer, we visualize the delivery of melanosome to keratinocyte in living cells. For melanosome, pigment globules were collected from culture medium of human primary melanocyte and were labeled with VybrantTM Cell labeling solution (DiI). The fluorescent melanosomes were co-cultured with primary keratinocyte and primary fibroblast, and then the melanosome transfer was visualized. DiI- positive bodies showed the similar behavior to those of melanosome shown by EM or immunocytochemistry reported previously. Namely uptake of DiI-positive bodies was already observed in the cytoplasm of the keratinocyte at 1hour after addition of the melanosome. The number and the distribution of the DiI-positive bodies around the peri-nuclear region were increased in the time-dependent manner until 24hr. Furthermore, the size and the distribution of the bodies were corresponded to those of human melanosome. These findings suggested that DiI-positive bodies are melanosomes and those could be phagocytosed through the cell membrane in our system. In addition, we found that primary fibroblast also uptook the DiI-positive bodies at 24hr after melanosome treatment. The live imaging in the co-culture system showed that pigmented globules tend to assemble around the peri-cellular region of keratinocyte, which is consistent with the phagocytosed melanosome. At present, we are performing the live imaging analysis in the co-culture using confocal microscope for unraveling the mechanism of melanosome transfer.

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Targeting protein trafficking pathways synergizes melanoma sensitivity to inhibition of the PI3K pathway and cytotoxic agents

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We recently developed a novel strategy for targeted melanoma therapy by targeting protein trafficking pathways in human melanoma cells to increase sensitivity to multiple cytotoxic agents by up to 10-fold. This targeted approach has the advantage of being independent of oncogene mutational status, and targets molecules that are functionally specific to melanoma and melanocyte cells, i.e. is largely cell-type specific. Here we show that this approach of inhibiting protein trafficking also influences human melanoma response to an inhibitor of signaling molecules, and provides synergistic effects between traditional cytotoxic therapies and treatment with signaling pathway inhibitors. When selected aspects of anterograde protein trafficking are inhibited by one of 2 methods, a) RNA silencing of either VPS33A or cappuccino (CNO) protein or b) cell surface signaling by exogenous addition of the agouti-signaling peptide derivative ASIP-YY, sensitivity to inhibition of the PI3K pathway is increased by 10-fold. When these inhibitory methods are used in combination together with either carboplatin or dacarbazine, cytotoxic agents currently used to treat advanced melanoma with little effect, sensitivity to PI3K pathway inhibition increases by up to 100-fold. Inhibition of anterograde protein trafficking also causes receptor tyrosine kinase (RTK) levels to decrease by 9-fold. Together, these results indicate that this targeting strategy can be useful for significantly increasing the efficacy and cytotoxicity of melanoma combinatorial treatments and to circumvent known melanoma treatment resistance mechanisms that rely on up-regulation of RTK expression.

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Insights into the etiology of Solar Lentigines through its MicroRNA and mRNA profile

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Solar lentigines are hyperpigmented lesions on photodamaged surfaces of the skin. They have been characterized histopathologically as having a hyperpigmented basal layer and elongated rete ridges. Here we examined the expression profile of microRNAs in lesional and non-lesional areas of skin to gain more insight into the etiology of these lesions. MicroRNAs (miRNAs) are small endogenous RNA molecules that play an important role in the regulation of gene expression. miRNAs have recently been shown to play a pivotal a role in diverse developmental and cellular processes and have been linked to a variety of skin diseases. Disruption of the miRNA expression has been shown to be involved in wound healing and inflammatory skin conditions. Through the analysis of over 160 samples via microRNA and mRNA arrays we have identified characteristic expression profiles in solar lentigines, distinct from that of photo-exposed skin. The microRNA data highlights the importance of the immune/inflammatory and cellular communication systems in the development and maintenance of solar lentigines. Target genes of differentially expressed microRNAs were confirmed by mRNA arrays and qPCR. Our mRNA array data confirms that ETBR, SILV and tyrosinase, which have been previously reported to be up-regulated in solar lentigines, are differentially expressed in lesions versus sun-exposed (peri-lesion) skin. We have also confirmed that some melanosome transfer genes and FGF-7 & FGFR2 are differentially expressed in perilesional skin compared to sun-protected skin. Through this approach we have identified novel genes and proteins involved in the etiology of solar lentigo.

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Melanoma differs from other cancers in generating a subset of DC-HIL$^+$ myeloid-derived suppressor cells (MDSC) that cause potent T cell suppression

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Cancers secrete tumor-derived factors (TDF) that induce MDSC to proliferate and potently suppress immune function. A prevailing concept, that phenotypic and functional differences among MDSC subsets reflect divergent interactions between tumors and host immunity, has not been proven. Having discovered DC-HIL to mediate MDSC suppressor function and their tumor-promoting effects in mice bearing melanoma, we asked whether a similar mechanism applies to other tumors. We examined growth of B16 melanoma, LL2 lung carcinoma, or EL-4 lymphoma implanted subcutaneously into wild-type (WT) vs. DC-HIL knockout (KO) mice. Melanoma growth was inhibited markedly in KO (vs. WT) mice, whereas lung carcinoma and lymphoma growth was unhampered in both mouse types. We assayed DC-HIL expression on MDSC in spleen of tumor-bearing mice. DC-HIL was expressed by 40% of MDSC in spleen or bone marrow (BM) of mice bearing melanoma, but absent completely on MDSC of LL2- or EL-4-bearing animals. Mice with melanoma generated 4 distinct MDSC phenotypes, with DC-HIL$^+$ restricted to the Gr-1^{med}/Ly6C^{high} subset solely capable of inhibiting T-cell activation triggered by melanoma Ag or CD3/CD28 costimulation. While present in LL2- or EL-4-bearing mice, this subset was DC-HIL^{neg} and (on a per-cell basis) 20-fold less-able to suppress T-cell activation and 5-fold less-efficient at inhibiting target-killing ability of cytotoxic T cells injected into naive mice. MDSC from all 3 tumors secreted inhibitory mediators (ROS, urea, NO) to about the same degree. Finally, we compared suppressor effects of DC-HIL$^+$ vs. DC-HIL^{neg} MDSC without TDF influence. MDSC were generated from *in vitro* culture of BM cells from WT or KO mice. DC-HIL$^+$ MDSC from WT mice inhibited T cell proliferation at a 10-fold greater level than DC-HIL^{neg} MDSC. Thus melanoma may be unique among tumors in its capacity to generate DC-HIL$^+$ MDSC with the most potent suppressor activity. Such a property may have evolved to counter melanoma's inherently strong immunogenicity.

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Single-cell analysis of mechanical properties of melanoma using deformability cytometry

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Mechanical biomarkers relating to cytoskeletal and nuclear structure and architecture can be important label-free identifiers of properties such as invasive or metastatic potential, phase of cell cycle, or activation state. A label free measurement of invasive potential may be important for determining prognosis and guiding treatment decisions. Deformability cytometry is a technique that utilizes microfluidic flows to create parallel external pressure on individual cells, combined with high speed microscopy to image the resulting deformation upon the cell. Cells with greater deformability are more flexible and have greater potential to cause metastatic seeding in patients with advanced cancer. We have fabricated an integrated device using inertial focusing and automated imaging analysis that combines these microfluidic flows in a high throughput platform that allows for measurement of over 2000 cells per second. This device was utilized to measure several patient-derived melanoma cell lines from patients with various stages of melanoma. We also included measurements from metastatic melanoma lines from patients who received vemurafenib, both before treatment began and after resistance to this drug developed. Our results indicated that cell lines from patients with higher tumor burdens (greater amounts of metastases) had greater deformability, consistent with the notion that cells with greater deformability have higher metastatic potential. Furthermore, melanoma cells from patient lines after vemurafenib resistance occurred had greater deformability compared with the same patients before treatment. Deformability cytometry offers the potential of a low cost and high throughput assay to complement conventional cytology methods and an adjunct method for determining invasive/metastatic potential of cancer cells that may be utilized for screening and monitoring of treatment efficacy.

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Fisetin inhibits human melanoma cell invasion through promotion of mesenchymal to epithelial transition and by targeting MAPK and NF-κB signaling pathways

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Malignant melanoma is a serious form of cancer responsible for approximately 80% of skin cancer-related deaths. Activating mutations in the serine/threonine kinase BRAF occur in 60-70% of malignant melanomas. Preclinical studies have demonstrated that BRAF plays an important role in regulating the MAPK signaling cascade in melanoma. The RAF-MEK-ERK (MAPK) pathway is the key regulator of melanoma cell invasion. In addition, activation of NF-κB via the MAPK pathway is regulated through MEK-induced activation of IKK. These pathways are receiving attention as potential targets for prevention/treatment of melanoma. In this study, we investigated the effect of fisetin on melanoma cell invasion and epithelial-mesenchymal transition, and delineated the molecular mechanism(s) underlying these effects. Using a Boyden chamber cell invasion assay with matrigel-coated membranes, we observed that treatment of malignant melanoma cells (A375, SK Mel 28 and RPMI 7951) with fisetin (5-20 μM) inhibited their invasion, which was associated with a decrease in the phosphorylation of MEK and ERK1/2. In addition, fisetin inhibited the activation of IKK leading to reduction in the activation of the NF-κB signaling pathway. Treatment of cells with an inhibitor of MEK (PD98059) or of NF-κB (CAPE) also inhibited melanoma cell invasion. Furthermore, fisetin treatment promoted mesenchymal to epithelial transition which was associated with a decrease in mesenchymal markers (N-cadherin, vimentin, snail and fibronectin) and an increase in epithelial markers (E-cadherin and desmoglein). Employing three dimensional skin equivalents consisting of A375 cells admixed with normal human keratinocytes embedded onto a collagen-constricted fibroblast matrix, we found that fisetin treatment reduced the invasive potential of melanoma cells. Based on these observations, we suggest that fisetin alone or in combination with ant-metastatic drugs could be useful for the management of melanoma invasion or metastasis.

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CD74 expression on human melanoma serves as a marker of progression and a target for apoptosis induction

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Based on the results of an inflammatory and autoimmunity gene PCR array, we have previously identified that a type II transmembrane glycoprotein, CD74 gene, is expressed in almost all subtypes of melanoma cells, including those with mutated BRAF and NRAS genes. CD74 associates with the MHC class II and it functions as a signal transducer upon binding with its ligand, macrophage migration inhibiting factor (MIF). In the analysis of a melanocytic tumor progression tissue microarray consisting of 480 tissue cores from clinically stratified melanocytic tumors, we observed positive staining (percentage score 1–3) for CD74 protein in 33.3% of benign nevi, 94.3% of primary melanoma tumor cells, and 95.9% of metastatic melanoma cells. Based on these observations, we performed an *in vitro* functional analysis, treating CD74 and MIF positive melanoma cells with ISO-1 ((S,R)-3-(4-Hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid), the CD74 antagonist. Our results indicate a downregulation of cell proliferation and also suppression of the expression of angiogenic factors IL-8 and VEGFα. Furthermore, treating melanoma cells with anti-CD74 carboyl-terminus antibody induced apoptotic cell death. These results suggest that CD74 functions as a communicator of the tumor microenvironment, supporting the downstream inhibition of apoptosis. Expression of CD74 is also reported in several other types of malignancies as a progression or prognostic marker and its targeting is also being considered as a therapeutic approach for CLL or Multiple Myeloma. In conclusion, CD74 appears as a promising novel target for the therapy of melanoma.

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***In vivo* mutation analysis and treatment of Gq mutated uveal melanoma**

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UvealMelanoma (UM) is the most common primary intraocular malignancy and the most common non-cutaneous melanoma in adults. Approximately 6 new cases per million population are diagnosed annually. Up to one half of patients develop metastasis invariably targeting the liver, and in some cases the lungs and bones, with a median survival of only 6-9 month with the onset of symptoms. Treatment of metastatic patients is unsatisfactory with very few improvements over the previous decades. Early detection of UM is key in designing effective treatments. Recent studies have identified GNAQ/GNA11 as a new oncogene in UM. Evidence suggests an important role of GNAQ/GNA11 in tumorogenesis. In humans, GNAQ or GNA11 mutations were detected in up to 83% of all primary UM, confirming study results. Facing the current absence of small molecules, able to restore GTPase activity of mutated G-proteins, we propose to use nanotechnology to detect and inhibit GNAQ/GNA11 mutations and signaling pathways. We have used very specific and sensitive nanoparticle-siRNA complexes to first detect GNAQ/GNA11 mutation due to the property of these nanoparticles to release fluorescent particles after binding to the transcripts of the mutated sequence and second to inhibit GNAQ/GNA11 RNA molecules. Nanoparticles prevented siRNA from degradation and facilitated the translocation into cells. We believe that nanoparticles can be used for treatment and detection of GNAQ/GNA11 uveal melanoma *in vivo*.

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Modulation of human skin pigmentation by various inhibitors of human tyrosinase

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Pigmented spots like lentigenes solaris, freckles and post-inflammatory hyperpigmentation are a major cosmetic concern. Therefore, many topical products are available, containing diverse active ingredients which reduce melanin production and distribution. Tyrosinase is the key regulator of melanin production and, therefore, the most prominent target for inhibitors of hyperpigmentary disorders. Many substances are described in the literature as inhibitors of tyrosinase, however, most of them were screened with mushroom tyrosinase and/or mouse melanoma cells and are, thus, poor inhibitors of human tyrosinase. We were interested in evaluating the inhibition of skin pigmentation by renowned compounds with skin whitening activity like hydrochinone, arbutin, kojic acid, and 4-butyl-resorcinol. We compared the inhibition of human tyrosinase activity in a biochemical assay as well as inhibition of melanin production in human melanocyte culture and melanoDermTM. Arbutin and hydrochinone are only poor inhibitors of human tyrosinase with an EC50 in the millimolar range, whereas kojic acid is ten-times more potent with an EC50 at 500 µM. By far the most potent inhibitor of human tyrosinase, however, was 4-n-butylresorcinol with an EC50 of 21 µM. Arbutin was least active in skin models with an EC50 for inhibition of melanin production > 5000 µM. Kojic acid inhibited with an EC50 > 400 µM. Interestingly, hydrochinone inhibited melanin production in melanoDerms with an EC50 around 40 µM, pointing towards a mechanism different from tyrosinase inhibition. 4-n-butylresorcinol, again, was the most potent inhibitor with an EC50 of 13.5 µM. *In vivo* efficacy of 4-butyl-resorcinol was tested in a clinical study. Elderly subjects with age spots treated twice daily two age spots with a formula containing 4-n-butylresorcinol and two control age spots with the corresponding vehicle. Within eight weeks 4-n-butylresorcinol visibly reduced the appearance of age spots while the control spots remained unchanged.

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Proline-rich tyrosine kinase 2 (Pyk2), a focal adhesion kinase (FAK) homologue, induces apoptosis in human malignant metastatic melanoma

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Overcoming apoptotic resistance while achieving selectivity in targeting apoptosis induction to cancer cells holds great promise for potential novel selective therapeutics for a life-threatening malignancy such as metastatic melanoma. Pyk2 is a non-receptor protein tyrosine kinase related to FAK. Pyk2 functions in a cell type- and cell context-specific manner to regulate proliferation, differentiation, migration, and survival. Here we report that endogenous Pyk2 protein is detected at similar levels in cultured human epidermal melanocytes (HEM) and in metastatic melanoma cells, and show that enforced expression of Pyk2 in human malignant melanoma cell lines A375 and A2058 induces apoptosis in cancer cells but neither in normal HEM, nor in normal human epidermal keratinocytes. Our data show that adenoviral-mediated expression of Pyk2 in A375 and A2058 cell lines results in effective killing of melanoma cells via a mitochondrial-based "intrinsic" apoptotic pathway, as evidenced by caspase-mediated cleavage of the known pro-apoptotic effectors poly(ADP)-ribose polymerase (PARP) and protein kinase C δ (PKCδ), decreased pro-survival factor STAT5 expression, and decreased phosphorylation/activation levels of FAK, Erk1/2 and Akt. Further analysis shows that similar apoptotic effects are achieved by overexpression of a truncated Pyk2 mutant that lacks both the N-terminal and the kinase domains of Pyk2, indicating that catalytic activity of Pyk2 is dispensable for apoptotic induction. These results suggest that Pyk2 mediates melanoma cell death as a scaffolding protein rather than a kinase, conceivably through interfering with FAK signaling implicated in the promotion of malignant phenotype. Ongoing studies are focusing on the molecular mechanisms of this regulation.

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Tenovin-1, a small-molecule inhibitor of SIRT1, imparts anti-proliferative response in human melanoma cells via p53 activation

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The incidence of melanoma, one of the most lethal skin cancers, continues to increase. Given that the currently available strategies are unable to effectively manage melanoma, more efficient target-based approaches are required to effectively treat this neoplasm. Ongoing studies from our laboratory suggest that SIRT1, a class III histone deacetylase, may be a promising target for melanoma therapy. Through its roles in a variety of physiological processes, SIRT1 has been shown to promote cell survival. In addition, SIRT1 has been shown to regulate p53 and FOXO transcription factors, both of which play a role in tumor progression. We have earlier shown that i) SIRT1 is overexpressed in melanoma cells relative to normal human melanocytes and ii) short hairpin shRNA- or Sirtinol-mediated inhibition of SIRT1 imparts anti-proliferative responses in melanoma cells (Nihal et al; In: AACR Annual Meeting: Proceedings; 2011 Apr 2-6, Orlando, FL Abstract #1647). To further explore the potential of SIRT1 as a therapeutic target in melanoma, here, we determined the effect of Tenovin-1, a more specific small molecule inhibitor of SIRT1 expression in multiple melanoma cell lines (A375, HS294T and G361) representing different stages of melanoma progression. We found that Tenovin-1 treatment resulted in a dose dependent decrease in SIRT1 protein levels that was accompanied by a significant decrease in the growth and viability of all types of melanoma cells employed. Interestingly, the anti-proliferative response of Tenovin-1 was concurrent with a concentration-dependent increase in the protein level of the tumor suppressor p53. Our data suggest that inhibition of SIRT1 via Tenovin-1 imparts anti-proliferative effects in melanoma cells by modulating the function of p53, which is rarely mutated in melanomas. Further in-depth studies are ongoing to confirm our results as well as to investigate the potential role of other SIRT1 targets, such as the FOXO transcription factors, in the biological response of Tenovin-1.

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BRAF mutation and FISH in the prognosis of cutaneous melanoma

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Correlating specific genetic alterations with melanoma prognosis may help to identify more aggressive tumor biology. It has been shown that chromosomal copy number aberrations detected by FISH may be prognostic in melanoma. Also, BRAF mutations are common in cutaneous melanomas (CM). The aim of this study is to compare the prognostic potential of BRAF mutation with other traditional prognostic markers used by the American Joint Committee on Cancer, alone or in combination with FISH. Along with the clinical course, Breslow depth, age, sex, site of disease, presence or absence of ulceration, mitotic count, and Clark's level were recorded. A total of 84 adult CM cases were evaluated of which 48 had metastasis. No statistical difference was found when comparing the frequency of BRAF mutations in the metastasizing vs nonmetastasizing CM groups. The prognostic power to detect metastasis in the presence of other traditional and molecular prognostic factors slightly increases when BRAF is combined with FISH using a probe targeting the MYC gene at 8q24 (c=0.76 for ulceration and Clark vs c=0.81 if MYC is added vs c=0.83 if MYC and BRAF are added). Our data suggest that the presence or absence of a BRAF mutation does not correlate with the likelihood for metastasis or disease progression in advanced melanoma. Moreover, these results confirm that other markers involved in cell cycle control may have more prognostic value in the setting of advanced disease.

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Inhibition of melanosome transfer by targeting d-dopachrome tautomerase

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The transfer of melanosomes from melanocytes to keratinocytes is a highly investigated method to modulate melanogenesis. Inhibition of melanosome transfer will lead to the accumulation of melanin within the melanocyte and this may activate a feedback loop to decrease overall melanogenesis. To further understand the underlying mechanisms which control pigmentation, we investigated the role of d-dopachrome tautomerase (DDT) on melanosome transfer. Although the physiological role of DDT is not fully elucidated, DDT shares a homologous amino acid sequence with macrophage migration inhibitory factor (MIF) and possesses similar tautomerase activity. MIF is a cytokine involved in inflammatory reactions and immune responses. DDT protein levels and activity have been shown to be elevated in UVB-induced inflammation in skin. Through the use of *in vitro* RNAi technology, knockdown of DDT message and protein in melanocytes has been shown to reduce the amount of melanosomes transferred to keratinocytes in a co-culture assay. RNAi results indicate that the role of DDT in melanosome transfer is restricted to melanocytes, since knockdown of DDT in keratinocytes does not impact melanosome transfer. DDT expression has been found to be up-regulated upon UV exposure and RNAi knockdown data provides supporting evidence for a potential role in skin pigmentation. Therefore, the enzyme was isolated and an *in vitro* assay established where DDT converts the non-physiological substrate d-dopachrome to 5, 6-dihydroxyindol through tautomerization and subsequent decarboxylation to identify select inhibitors. These compounds were subsequently evaluated both in co-culture assays and living skin equivalents containing melanocytes. Melanosome transfer and overall pigmentation were decreased in both assays respectively. These and other results to be presented collectively demonstrate the *in vitro* activity of DDT inhibitors on decreasing pigmentation and suggest that topical applications may modulate pigmentation *in vivo*.

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Dermoscopic patterns serve to identify underlying growth promoting mutations in melanoma

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Melanomas are classified as superficial spreading(SSM), lentigo maligna(LMM), acral lentiginous(ALM), and nodular(NM). Sun exposure has also been used to classify. Genetic analysis has revealed BRAF, NRAS, and KIT as major mutually exclusive mutated growth promoting genes. However, for a significant fraction of melanomas the growth promoting mutation is unknown. BRAF is prevalent in SSM/intermittent-sun whereas KIT is prevalent in LMM/chronic-sun-exposed melanomas. However, these mutations do not fully correlate with current classification systems. Dermoscopy improves visualization of growth patterns and may better segregate molecular subtypes. We hypothesize unique dermoscopic patterns are caused by specific underlying growth promoting mutations. For this study we chose 4 patterns; negative network, rhomboidal, polygonal, and dark homogenous streaks. Three melanomas in each group were subjected to laser capture microdissection, melanoma and keratinocyte DNA isolated, and analyzed for BRAF, NRAS, HRAS, KIT and GNAQ mutations. The dark homogenous streaks pattern revealed KIT exon 17 mutations in melanoma. All these mutations were within or adjacent to the catalytic region of the second kinase domain. The expected frequency of exon 17 KIT mutations in melanoma is 0.19%. The chance of this occurring in 3 tumors prospectively segregated is 1 in 146 million; highly suggestive that the mutation is correlated with the pattern. Despite the high prevalence of BRAF and NRAS mutations, the other patterns were wild type for the mutations tested suggesting that BRAF and NRAS may be enriched in more advanced tumors. In summary, our results indicate that dermoscopic patterns reveal melanomas that lack common mutations and therefore may allow for the identification of new driving mutations. Furthermore specific dermoscopic patterns can be identified that correlate with specific gene mutations.

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Biopsy performance of a multi-spectral computer vision system for melanoma compared to physicians

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This reader study was designed to compare the biopsy performance (i.e., sensitivity and specificity) of an automatic, objective, non-invasive, multi-spectral computer vision system (MSCVS) to that of dermatologists, including general dermatologists and pigmented skin lesion experts, as well as that of primary care physicians. Physicians from each discipline were randomly recruited as readers for this cross-sectional study. Participating readers evaluated 130 online cases (65 melanomas and 65 non-melanomas), including photographs and clinical history, to determine their biopsy performance. Sensitivity and specificity of both the physicians and the MSCVS were estimated by comparison to a dermatohistopathologic reference standard. 155 eligible physician readers (46 GDs, 64 PSLEs, and 45 PCPs) participated. Biopsy sensitivity of the MSCVS (0.97 ± 0.02; 95% CI 0.90 – 0.99) was significantly higher than that of GDs (0.73 ± 0.03; 95% CI 0.67 – 0.80), PSLEs (0.71 ± 0.03; 95% CI 0.65 – 0.77), and PCPs (0.71 ± 0.03; 95% CI 0.64 – 0.78), p<0.0001. Biopsy specificity of the MSCVS (0.09 ± 0.04; 95% CI 0.04 – 0.19) was lower than that of GDs (0.51 ± 0.04; 95% CI 0.43 – 0.60), PSLEs (0.50 ± 0.04; 95% CI 0.42 – 0.58), and PCPs (0.45 ± 0.04; 95% CI 0.37 – 0.53), but was consistent with that of the most sensitive physicians. This study is the largest and most comprehensive study to date comparing the biopsy performance of the MSCVS to that of clinicians. Our results suggest that the MSCVS holds promise as a highly sensitive adjuvant tool for the early detection of melanoma.

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Side population cells from human melanoma tumors reveal diverse mechanisms for chemoresistance

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Side population (SP) is identified as cells capable of excluding the fluorescent Hoechst dye and anticancer drugs, and represents hematopoietic stem cells and chemoresistant cells from several solid tumors. In this study, a direct *in vivo* xenograft model was generated to maintain tumor microenvironment and hierarchical structures. We confirmed the presence of SP cells in patient melanoma tumors and xenografted tumors. Gene expression and protein analysis revealed overexpression of ABCB1 and ABCB5 in SP cells. We demonstrated that melanoma SP cells were resistant to paclitaxel, a substrate of ABCB1, both *in vitro* and *in vivo*, and that silencing ABCB1 or ABCB5 by siRNA sensitized cells to paclitaxel treatment. Interestingly, melanoma SP cells were also resistant to temozolomide, which is not a substrate for ABC transporters, through IL-8 upregulation, and silencing IL-8 by siRNA sensitized cells to temozolomide treatment. Whereas silencing either gene (ABCB1, ABCB5 or IL-8) reduced SP cell numbers, silencing ABCB1 or ABCB5 did not affect IL-8 expression and silencing IL-8 did not affect ABCB1 or ABCB5 expression, suggesting that chemoresistance of SP cells derives from at least two independent mechanisms: one from transporter-conferred resistance and the other from intrinsic resistance. In addition, gene profiling identified NF-κB, α6-β4-integrin and IL-1 signaling networks as differentially upregulated in melanoma SP cells, and there was a significant increase of PCDHB11 and decrease of FUK and TBX2 in these cells. In conclusion, we provide evidence that SP is an enriched source of chemoresistant cells in human melanomas, and suggest that selected genes and pathways of SP cells may be an important target for effective melanoma therapies.

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Dysregulation of the unfolded protein response contributes to chemoresistance in melanocytes

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Adaptation to endoplasmic reticulum (ER) stress is thought to be a key factor in melanoma progression and chemoresistance. Hypoxia during melanomagenesis causes oxidative stress and buildup of misfolded proteins in the ER, which triggers the unfolded protein response (UPR), an intricate mechanism that enables cells to recover from ER stress. Failure to restore ER homeostasis can trigger apoptosis. Evading UPR-mediated apoptosis may facilitate survival of cancer cells. Melanocytes lacking expression of the Oculocutaneous Albinism Type 2 (Oca2) gene adapt to sustained UPR activation induced by accumulation of the enzyme tyrosinase in the ER, remaining viable. Oca2 is the fifth most frequently downregulated gene in melanoma. We therefore investigated the mechanisms that contribute to survival of Oca2-null melanocytes despite UPR activation. The UPR consists of three signaling pathways: Ire1 (primarily promotes survival), Perk (promotes cell death), and Atf6. UPR activation was compared in wildtype and Oca2-null melanocytes. When treated with thapsigargin to induce additional ER stress, Ire1 expression and Xbp1 splicing increased indicating UPR activation. Typically, UPR activation also leads to Perk-mediated phosphorylation of eIF2α. Remarkably, eIF2α was rapidly dephosphorylated in Oca2-null cells, disrupting pro-apoptotic Perk signaling and preventing increased expression of the downstream effector Atf4. This phenomenon was mediated by Gadd34; treatment with guanabenz, a specific inhibitor of the Gadd34-PP1 eIF2α phosphatase, blocked eIF2α dephosphorylation during thapsigargin induced ER stress. Furthermore, viability of guanabenz and thapsigargin treated Oca2-null melanocytes was decreased compared to cells treated with thapsigargin alone. Our results suggest that Oca2-null melanocytes adapt to persistent ER stress by UPR dysregulation, mediated by disruption of the pro-apoptotic Perk signaling pathway via eIF2α dephosphorylation.

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Interferon-gamma and tumor associated macrophages in human melanoma patients

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A pilot study of primary malignant melanoma patients was initiated in order to investigate whether IFN-γ-produced by macrophages occurs in humans as has been proposed in a murine model. In a UVR-initiated melanomagenesis mouse model, IFN-γ produced by cutaneous macrophages after UVB irradiation enhanced malignant melanoma tumor growth. Blockade of IFN-γ abolished the enhanced melanoma proliferation. Biopsy specimens of fresh-frozen primary melanoma tissue were microscopically analyzed with confocal immunofluorescence for macrophage infiltration and colocalization of macrophage-derived IFN-γ. Preliminary data shows a subset of tumor infiltrating CD68 positive macrophages colocalized with IFN-γ (Pearson's Correlation = 0.31 ± 0.05). Additionally, a population of infiltrating CD4 positive lymphocytes strongly colocalized with IFN-γ as well (Pearson's Correlation = 0.65 ± 0.12). In a separate case-control study of patients with a history of melanoma, macrophage infiltration and activation in response to UVR is being analyzed. Preliminary results show that relative to healthy volunteer skin, melanoma patients have an increased level of dermal infiltrating CD68 positive macrophages (2.37 fold increase when normalized to non-irradiated skin). Current findings verified that macrophages are producers of IFN-γ and that melanoma patients have a differential macrophage response to UVR. The long-term benefit of these studies will be to provide quantitative data about the presence of pro-tumorigenic macrophage-derived IFN-γ, and to potentially build a rationale for future immune-modulatory studies for the prevention or treatment of human malignant melanoma.

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Dual inhibition of AkT and mTOR by fisetin induces tumor regression in a 3-D melanoma model

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Curative treatments for metastatic melanoma remain elusive with the median survival time less than a year for patients diagnosed with the metastatic disease. The Akt/mammalian target of rapamycin (mTOR) signaling pathway, with a central role in physiological processes such as survival, apoptosis and autophagy, is known to be aberrantly upregulated in many types of cancers including melanoma where it negatively influences disease prognosis. We recently showed that the dietary flavonoid fisetin inhibited melanoma cell growth in monolayer cell cultures and xenograft mouse model (Syed et al., JID 2011). Here, we investigated the effect of fisetin employing a reconstituted 3D organotypic melanoma skin model comprising of A375 melanoma cells, epidermal keratinocytes and dermal fibroblasts that recapitulates stage-specific properties of melanoma growth. The 3D melanoma cultures were treated with fisetin (80 μM) for 16 days and cross-sections were taken at four day intervals. H&E-stained images of fisetin-treated reconstructs exhibited significantly less melanocytic lesions in sharp contrast to the untreated reconstructs which showed nests of tumor cells and many invading disseminated cells. Immunohistochemical analysis demonstrated a significant decrease in the phosphorylated forms of Akt and mTOR and the downstream target p70S6K in the treated versus the control sections. Western blot data obtained from fisetin-treated melanoma monolayer cell cultures showed a decrease in the protein expression of PI3K and inhibition of phosphorylation of Akt, mTOR, p70S6K, eIF-4E and 4E-BP1. In cell free biochemical assays fisetin inhibited mTOR kinase activity in an ATP-competitive manner. Xenograft studies in nude mice implanted with 451Lu melanoma cells showed decreased Akt and mTOR phosphorylation in tissue sections of fisetin-administered animals. Collectively, our data suggest a key role of Akt and mTOR in melanoma progression and identify fisetin as a modulator of these targets. Fisetin can therefore be a useful agent for slowing the progression of melanoma.

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Neutrophilic dermatoses associated with vemurafenib

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Vemurafenib, a small molecule inhibitor of BRAF kinases bearing the V600E mutation was recently approved by the FDA for use in advanced melanoma. Although cutaneous eruptions are listed among the drug's potential side effects, they are not clinically or histologically characterized. Herein we describe clinical and histologic findings from three patients who developed various neutrophilic dermatoses while on vemurafenib. All three patients developed acneiform papules and tender, erythematous nodules within days of beginning vemurafenib therapy. In two patients, these findings were accompanied by markedly elevated inflammatory markers. Histologic examination revealed findings ranging from neutrophilic infiltrates with vasculitic changes to combined septal and lobular panniculitis. One patient also developed the papillomas and photodistributed erythema observed in previous studies. These cutaneous eruptions subsided rapidly with cessation of vemurafenib, and only recurred at a reduced dose in one patient. Vemurafenib can cause diverse cutaneous eruptions, ranging from acneiform papules to panniculitis. These eruptions may be neutrophil-mediated, and appear to be accompanied by marked systemic inflammation. Further exploration into understanding the mechanisms behind these cutaneous effects is warranted.

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Genital melanocytic nevi in children

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Melanocytic nevi on genital skin are relatively uncommon and often concern patients, parents, and physicians. Furthermore, genital nevi are more likely than nevi on other sites to demonstrate atypical histological features despite clinically benign behavior; thus, biopsy may lead to unnecessary apprehension and excisions in an anatomically-sensitive area. To improve our clinical understanding of genital nevi in children, we conducted a retrospective analysis of all patients aged 0-18 years diagnosed with melanocytic nevi at the NYU faculty practice between 2000-2010; subjects with genital nevi were contacted for additional longitudinal data. Genital nevi were defined as small or medium melanocytic nevi on the scrotum or penis in boys or clitoris, clitoral hood, labia minora/majora in girls. In our referral population, the prevalence of genital nevi among patients seen for nevi was 3.5% (40/1159) and was similar in boys (57.5%) and girls (42.5%). Most genital nevi (13/19) developed between 0-2 years. No association was found between the presence of genital nevi and acral/scalp nevi, "moleyness", family history of dysplastic nevi or of melanoma. The most common dermoscopic pattern was globular (14/15). Genital nevus was the chief complaint in 67% of cases, supporting the observation that genital nevi are a source of concern to families and referring physicians. Family preference resulted in biopsy of 2 genital nevi, which were congenital- pattern compound nevi. In contrast, 3 genital nevi were biopsied due to clinician concern, which included a compound dysplastic nevus, a recurrent Spitz nevus and a congenital pattern nevus combined with epithelioid Spitz nevus of moderate atypia. No cases of melanoma were diagnosed. Our study provides data regarding the clinical characteristics, anatomical locations, and dermoscopic patterns of genital nevi in children and demonstrates that there is no apparent relationship between genital nevi and moleyness, or family history of dysplastic nevi or melanoma.

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Detection of melanoma by systemic administration of fluorocoxib and wide-field fluorescence imaging

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Melanoma is a deadly disease accounting for 75% of all skin cancer related deaths. Early detection is crucial given that the survival rate reaches 95% if caught before lesions are 1 mm in diameter. Taking advantage of the high levels of cyclooxygenase-2 (Cox-2) expressed in melanoma tumors and the ability of the fluorescence probe, LM-4777 (fluorocoxib), to preferentially target Cox-2, we have been able to detect melanoma cells by non-invasive whole-body imaging in living mice. Fluorocoxib was administered systemically into SCID hairless mice carrying subcutaneously injected melanoma (LOX cells) and HCT116 (Cox-2 negative) cells, and the mice were imaged 3 h later using spectral unmixing. The detection sensitivity allows for visualization of ~1000 melanoma cells above background. The fluorescence intensity from 1 million melanoma cells was about 40-fold over background and more than 2-fold higher than the signal intensity from 1 million HCT116 cells. Melanoma tumor xenografts (~10 mm in diameter) were detected showing a 6-fold higher signal over background. The presence of Cox-2 in melanoma xenografts was further demonstrated by immunostaining of tissue sections. Fluorocoxib and *in vivo* fluorescence imaging may be a useful methodology for early detection of melanoma and could be used as a guidance marker for margin detection and image-guided resection.

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Inhibition of FICZ-induced pigmentation through an AhR antagonist towards NHEK

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6-formylindolo[3,2-b]carbazole (FICZ) was used as a xenobiotics and incubated with NHEK. When conditioned media from NHEK exposed to FICZ was transferred to human melanocytes, we have previously measured and reported an increase in melanin content. Here, we show that the addition of this xenobiotics to NHEK increased CYP1A1 transcription, therefore showing AhR activation. We then incubated an AhR antagonist, baicalin, with NHEK-FICZ and transferred in the same way this conditioned media to human melanocytes. A 22%, 15% and a 4% decrease in melanin content was measured with 100, 50 and 10uM baicalin, respectively. Many signal transduction pathways contribute to the regulation of melanogenesis. In this study, we used a two cell system, treating keratinocytes with an AhR agonist+/- the antagonist and transferring to human melanocytes. Here, we show that AhR activation by xenobiotics in NHEK can indirectly increase melanogenesis in human melanocytes. By using an AhR antagonist on NHEK, we are reducing this induction of melanin.

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Distinct gene expression signatures classify desmoplastic and nodular melanomas

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Desmoplastic melanoma (DM) is a unique variant of cutaneous melanoma characterized by the presence of malignant spindle cells dispersed in a prominent collagenous stroma. Both the clinical behavior and histologic appearance of DM differ from conventional melanoma. As an approach to identify new diagnostic biomarkers and to understand its unique biology, we have utilized whole genome expression analysis using RNA from fresh frozen homogenous melanoma tissue. Specifically, frozen tissue in volumes of 2mm3 were macrodissected from sections containing >70% tumor and isolated for total RNA. Signatures generated using Affymetrix HuGene 1.1ST array were compared between 5 DM and 5 nodular melanomas (NM) of matched depth. As quality measures, replicate analysis of variance between batches and within paried samples demonstrated a high concordance ($R2 = 0.99$). Using unbiased clustering anaylsis revealed two separate signatures associated with DM vs. NM. As expected, genes associated with melanocytic differentiation (MITF, SILV, TYR) are upregulated in NM as compared to DM (differential expression 7 to 37 fold higher) whereas genes associated with stemness and neuroectoderm are enriched within DM including NGFR, claudin1, jagged1. Further immunohistochemical and RT-PCR analysis is underway to verify these gene signatures.

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The dichotomous effects of IL-17 and TNF on normal human melanocytes may contribute to both hyper- and hypo-pigmentation associated with skin inflammation

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Activated Th17 T-cells have been identified in vitiligo and conflicting data suggest Th17 T-cells/IL-17 may promote or inhibit melanogenesis and melanoma growth. IL-17 may cooperate synergistically with TNF in activating target genes in different cell types. In order to determine how human melanocytes respond to IL-17 and TNF, we treated primary human epithelial melanocytes cultured in serum-free media with IL-17 alone, or in combination with TNF. Morphologically, melanocytes aggregated and formed clusters over 48hrs. In terms of target gene expression, qPCR showed that IL-17 can induce mitogenic cytokines in melanocytes (IL-6, IL-8, CXCL1, etc.). Such cytokine induction was verified at the protein level by FACS-based intracellular staining and ELISA, and IL-17 synergizes with TNF to promote the induction of IL-8 and CXCL1. Another intriguing effect of IL-17 and TNF was the inhibition of genes essential to melanocyte lineage and pigmentation signaling (Tyr, Dct, Mitf, SNAI2, etc.), which were downregulated both in qPCR data and microarray data. To better understand the *in vivo* impact of these findings, we studied skin lesions of psoriasis vulgaris, as high expression of IL-17 and TNF in this condition has been established. We found a significant increase in melanocyte number by immunohistochemistry with Melan-A in psoriasis lesions vs. non-lesional skin ($p<0.05$). Meanwhile, qPCR analysis of lesional psoriasis biopsies showed downregulation of genes specific to pigmentation pathways. Taken together, this study has demonstrated the dichotomous effect of IL-17 and TNF, which not only activates mitogenic cytokines for melanocyte proliferation but also inhibits melanogenesis by downregulating the genes important to pigmentation pathways. These findings might reveal mechanisms underlying both hyper- and hypo-pigmentation associated with skin inflammation in diseases such as psoriasis.

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Role of microRNA-211in melanocytes and melanoma

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MicroRNAs (miRNAs) are small non-coding regulatory RNAs that regulate gene expression at post-transcriptional level. Evidence is accumulating that miRNAs are involved in many physiological process such as differentiation, proliferation and apoptosis and in almost all aspects of tumor biology including tumor cell survival, proliferation and migration. miRNA-211, which maps to the sixth intron of TRPM1 gene, is expressed abundantly in primary melanocytes and its expression is reduced in primary and metastatic melanoma cell lines. Expression of miRNA-211 correlates with expression of TRPM1, a transient receptor potential family member calcium channel protein that we showed to be involved in regulation of melanocyte calcium homeostasis and melanin pigmentation. Here, we show that inhibition of miRNA-211 expression by antago-miR-211 inhibited Ca+2 uptake and the growth of rapidly growing melanocytes. Ectopic expression of miRNA-211in melanoma cells inhibits growth and reduced their migration. Regulation of miRNA-211 and its function in melanocytes and melanoma have not been well understood. In this study, we show that ectopic expression of p53 (a tumor suppressor) in melanocytes increase miRNA-211 expression and decrease TRPM1 expression. Inhibition of miRNA-211 did not affect TPRM1 expression and knockdown of TRPM1 did not change miRNA-211 levels in melanocytes, suggesting lack of feedback regulation in TRPM1 and miRNA-211. Using RAB22A 3′-UTR luciferase reporter and site-directed mutagenesis, we show that RAB22A (protein involved in trafficking of and interaction between endosomal compartments) is a miRNA-211 target and show that antagomir-mediated suppression of miR-211 decreases RAB22A mRNA expression in melanocytes. Using live cell imaging, we demonstrate that inhibition of miRNA-211 in melanocytes inhibits/retards vesicular trafficking in melanocytes. We propose that miRNA-211 plays an important role in intracellular vesicular trafficking and may explain the effects of ectopically expressed miRNA-211 in melanoma.

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A mouse model of vitiligo with spontaneous epidermal depigmentation

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A HLA-A2 restricted, tyrosinase reactive T cell receptor derived from CD4+ TIL was cloned, characterized and transgenically expressed in HLA-A2 transgenic C57BL6 mice under the TCR promoter. Resulting h3TA2 animals were crossed with k14-SCF mice that carry epidermal melanocytes. Surprisingly, transgenic T cells are found in the periphery of h3TA2 mice and spontaneous depigmentation of the pelage is observed, accompanied by loss of melanocytes and T cell infiltration to the skin. TCR Tg expression is found primarily on T cells expressing a CD4−CD8− phenotype, specifically activated in response to HLA-matched human and mouse melanocytes. A five-fold reduction in infiltrating Treg was observed in skin homogenates but not splenocytes from h3TA2 mice, similar to reduced Treg abundance selectively in human vitiligo skin. On a k14-SCF background, we observed much more rapid depigmentation of the skin and pelage amounting to 75% (versus 42%) depigmentation by 25 weeks of age and 80% (versus 60%) depigmentation by week 28. Depigmentation is strikingly symmetrical as in human vitiligo and complete, in contrast to hair greying observed in other models. Curiously, depigmenting mice developed nevus-like skin lesions at around 3–4 weeks old, which increased in pigmentation level over time until stabilizing at 7–8 weeks. Intensely pigmented lesions contain epidermal melanocytes and are infiltrated by T cells, suggesting that resilient melanocytes undergo post-inflammatory hyperpigmentation. The NeVi (Nevus-Vitiligo) mouse faithfully reproduces the effector phase of autoimmune reactivity in vitiligo and can be used to assess efficacy of new treatment modalities for the disease.

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New dynamic mechanisms of melanogenesis in each pattern of vitiligo patients treated with NB-UVB phototherapy

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The differences between each repigmentation pattern (follicular, diffuse and marginal) in vitiligo have not been elucidated. To clarify this, we included 11 patients with vitiligo diagnosis who were in session number 15 of UVBnb [311nm] therapy. 5 biopsies were taken from the trunk of each patient from normal skin, unresponsive macule and each repigmentation pattern in the edge of the macule. The dynamics were analyzed throughout immunofluorescence with a confocal microscope utilizing 4 antibodies: CD200 (stem cells), SXO9 (melanocytes differentiation), C-Kit (melanocyte proliferation) and pFAK125 (melanocyte migration). Results show that in the 3 repigmentation patterns predominate a different mechanism of melanogenesis. The follicular repigmentation pattern shows that stem cell from the hair follicle differenciate to melanocytes. In the diffuse pattern, it predominates the peripheral melanocyte migration into the macule. Marginal repigmentation displays melanocyte proliferation while the unresponsive macule exhibits static melanoblasts. Our results have a $p=0.001$ from ANOVA and the honest significance by Tukey's method was used. With this research, new dynamic mechanisms of melanogenesis in vitiligo have been described. These findings contribute to better understanding the physiopathogenesis of vitiligo and open a wide door for further research of new therapies for its treatment.

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Deficiency in IL-23, but not IL-12, promotes the development of melanocytic tumors with regional lymph node involvement in C3H/HeN mice

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Genetic and environmental factors act in concert to deregulate melanocytic stem cells ultimately leading to their transformation and progression into metastatic melanoma. Treatment of C3H/HeN mice with dimethylbenz(a)anthracene (DMBA) followed by TPA induces the formation of dysplastic pigmented lesions, but not melanoma. Because IL-12 and IL-23 have been shown to play a critical role in other types of tumors, their role on the formation of pigmented lesions and their progression to melanoma was investigated using C3H/HeN mice deficient in IL-12 (p35-/-), IL-23 (p19-/-), or both IL-12/23 (p40-/-). Panels of mice were treated with DMBA followed by twice weekly doses of TPA. IL-23 deficiency was found to promote the initiation of pigmented lesions by 50% ($p\leq0.001$) and their radial growth by 100% ($p<0.05$) compared to wild-type (WT) mice. In contrast, there were fewer pigmented lesions in p35-/- and WT mice than p40-/- mice. Mouse melanocytes from WT mice were also found to upregulate IL-23R expression in response to IL-23 but not IL-12, providing evidence that IL-23 can act directly on melanocytes. Mice subjected to the DMBA/TPA protocol were found to have nests of melanocytes in their draining lymph nodes when examined histologically. Eighty percent of the LNs obtained from IL-23 KO mice were able to generate melanocytic cultures from their lymph nodes. In contrast, only 33% of the lymph nodes from IL-12 KO mice were able to do so. Our findings suggest a novel role for IL-23 in suppressing development of pigmented nevi and migration of melanocytc cells to regional lymph nodes.

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Efforts to provide a comprehensive overview of genetic changes in canine melanoma as a means to identify genetic correlates for drug response

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One major obstacle to the advancement of personalized medicine has been the difficulty in identifying correlates of response against a varied human genetic background. A solution to this dilemma is to use spontaneous canine disorders to model human diseases. The intent of this study is to leverage the low genetic diversity of purebred dogs coupled with breed predilections for melanoma to identify the underlying genetic lesions and sensitized biochemical pathways that contribute to canine melanoma. Unfortunately, the molecular pathogenesis of canine melanoma and its relevance to its human disease counterpart is poorly understood. We are undertaking a comprehensive study of canine melanoma including copy number analysis, and sequencing. We report on the genomic alterations in 37 canine melanomas using the Illumina CanineHD whole genome genotyping arrays. We identified 1233 copy number aberrations in this cohort (average of 31 per genome). Among 780 identified regional amplicons we noted those harboring the known human oncogenes including KRAS; (n=6), BRAF (n=5). A total of 453 regional deletions were observed. In human tumors, including melanoma, tumor suppressor genes are frequent targets of homozygous deletions (HD). In this cohort we identified a total of 198 HDs including those targeting tumor suppressor genes deleted in human melanomas, such as PTEN; (n=3) and CDKN2A (n=4). In addition we have sequenced >4,100 exons in this cohort spanning more than a dozen genes known to be mutational targets in human melanoma including BRAF, CDKN2A, c-KIT, etc. Our results are beginning to provide a genome-wide view of canine melanoma and we will highlight similarities with human melanoma underlying the shared pathogenesis between the two species.

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Keratinocyte p53, skin tanning and melanoma

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p53 in keratinocytes is a major target of UV irradiation and is a master regulator of the skin sunburn response. p53 has been shown to stimulate expression and release of pro-melanogenic cytokines alpha-MSH, KITLG and ET1 from keratinocytes after UV exposure. These paracrine factors are critical mediators of skin tanning, including increased melanocyte pigment synthesis and recruitment of melanocytes to the burned skin area. We hypothesize that p53 activation in keratinocytes provides a strong signaling environment that assists in establishing and promoting pigmented lesions at the very earliest stages of their development. To test this hypothesis, we have modeled the p53 dependent skin tanning response using the Sooty Foot Ataxia (SFA) mouse that carries a ribosomal protein haploinsufficiency allele that results in p53 hyperactivation. The SFA mouse presents with hyperpigmented footpads, ears and tail. These phenotypes are a result of increased melanogenesis and melanocyte number. We examined in detail the skin gene expression in SFA, WT and p53 null mice to identify the key p53 responsive pro-tanning growth factors. Moreover, we performed DMBA and UV carcinogenesis studies on SFA mice and observed an increase in pigmented lesions with some of these lesions undergoing conversion to melanoma. This mouse model strongly supports the idea that skin tanning and pigmented lesion formation are intimately linked, largely through a melanoma promotional activity of keratinocyte p53.